

Reactors, enriched uranium and NPT

The United States should think again about its decision not to supply enriched uranium for a German research reactor, which gives a poor impression in advance of next year's non-proliferation conference.

THE multi-faceted row that has blown up in Germany over the nuclear fuel for a new research reactor at Garching, near Munich, will have echoes well outside Bavaria (see page 89). Indeed, it raises a principle that will inform, and possibly inflame, the conference next year at which the Non-Proliferation Treaty (NPT) will be reviewed, possibly for the last time if its current members cannot agree on the terms on which it should be continued. For what is happening will raise in the minds of many non-nuclear governments the question of whether the nuclear powers that sponsored the NPT are serious about their undertaking to provide technical assistance for peaceful projects undertaken by signatories of the treaty. It is, of course, ironical that the victim in this case should be technically advanced and law-abiding Germany.

There is, of course, nothing in the NPT to prevent the use of highly enriched uranium fuel in civil reactors (that at Garching is meant to generate neutrons for research), even though the same material could be used for making bombs. The assumption (and the solemn obligation of the signatories) is that the material will be subjected to whatever safeguards are needed to ensure that it is not diverted to military uses.

The unwillingness of the United States to supply Germany with the fuel it wants stems from domestic legislation — the Nuclear Non-Proliferation Act (NNPA) going back to President Jimmy Carter's time. That was when US electricity utilities, faced with new high costs for reprocessing uranium fuel, decided that it might be more economical to store it unprocessed against the revival of the market in fissile material that has not yet materialized. So why not make a virtue out of economic prudence by requiring that nuclear trading partners of the United States should similarly avoid reprocessing? And why not go one administrative step further, and decline to supply fissile material that could be used for making bombs?

To be fair, in declining to supply Germany with enriched fuel, the US government is faithfully echoing the spirit of the report of the National Academy of Sciences committee that earlier this year came close to recommending that the United States should use its whole influence to prevent the physical production of plutonium and enriched uranium elsewhere in the world. The common interest in non-proliferation notwithstanding, there are good grounds for believing that it would instead be preferable to face up to the difficulties of providing international custodianship for fissile material (see *Nature* 367, 301–302 & 307; 1994), which will be

needed anyway. But the issue that faces Germany now, and which is certain to arise at the review conference next year, is whether it is equitable that paid-up members of the NPT should be subjected to requirements in the conduct of their peaceful nuclear business that go beyond the treaty proper.

In reality, there are two serious objections. First, it appears to have been forgotten in the United States that the NPT is meant to be a bargain between nuclear and non-nuclear powers. In return for self-denial on nuclear weapons, non-nuclear states would be given (again under international safeguards) technical assistance in peaceful nuclear energy. In practice, there has been very little business since the NPT was negotiated in the late 1960s, but that does not change the principle. The non-nuclear members of the NPT will rightly ask what has happened to the other side of the bargain they signed at the review conference next year. But there is another danger. If even countries such as Germany are unable to rely on international trade in nuclear energy, will that not confirm them in the belief that self-sufficiency is essential? And will that not eventually imply a proliferation of enrichment and reprocessing plants?

For the nuclear powers and all those with an interest in avoiding the spread of nuclear weapons, next year's NPT conference will be a crucial proceeding. The most serious danger, of course, is that those who have found the treaty so far to have been an irksome burden (and an extra cost) will defect. Germany is unlikely to be one of them, whatever happens about the fuel for Garching. But the principle of the ordinary marketplace, that those who have struck a bargain have a right to expect that their entitlements as well as their obligations will materialize, applies (or should apply) to international treaties as well. The entitlements cannot be wished away by administrative action elsewhere. □

Education gains

American children are called "prisoners of time" in a report comparing US schools to Germany and Japan.

ONCE again, education is edging close to the forefront of political attention in the United States and elsewhere as various groups point to obvious flaws in the way children are now taught basic subjects, science and mathematics included. On education in its broadest sense, the National Education Commission on Time and Learning (created by



Congress under the Education Council Act 1991) has issued a scathing review of the way US schools have dropped core academic subjects in order to fit intellectually marginal courses, such as sex education and parenting, into the school day. The commission says US schools have followed an unspoken rule that says: "learn what you can in the time we make available". That implies that students too often graduate without learning what they need to know in history, science, mathematics, even the English language.

US schools typically provide only 5° hours of classroom time a day, and for a mere 180 days a year. (The schedule derives from the previous century, when students had to get home to work on the family farm.) This outmoded educational pattern compares unfavourably with that in Germany, France, Britain and Japan. In Germany, for instance, the total time devoted to the study of core subjects is 3,528 hours a year, but in the United States it is only 1,460 hours. Furthermore, the commission found an important difference in attitude between school systems in the United States and elsewhere. Bluntly put, it says that "in Germany and Japan, learning *matters*." By contrast, social problems (particularly in the inner cities) have turned too many US schools into the equivalent of day-care centres.

Just as the National Commission published its analysis of US schools generally, together with its sweeping recommendation for altering the pattern of US society (and of US television programming) by substantially lengthening the school day, the US National Science Foundation (NSF) announced a pilot project to revamp science education in nine cities, each of which will receive \$15 million over five years (see page 87). On top of that, Bruce Alberts, the molecular biologist in his first year as president of the US National Academy of Sciences, has declared improved science education nationwide to be one of his most important goals; he is exploring several ways in which the academy can engage scientists in teaching in local school systems. (With an impressive project along these lines in San Francisco to his personal credit, he is exploring ways of directing the academy's intellectual talent into new approaches to teaching science.) And the Howard Hughes Medical Institute, which spends most of its money on research, is also supporting innovative science programmes at undergraduate colleges.

So are we back in the *Sputnik* days, when fears of Soviet domination in space prompted a huge investment in science and mathematics curricula? Hardly. That was made to seem a crisis, but the National Education Commission's report shows that present difficulties have their roots in long-established attitudes to schooling and to education generally. In any case, the threat now is industrial competitiveness. And for what it is worth, the United States is not alone in its sense of being educationally deprived; only this week, the British government has been fine-tuning its national curriculum (and paradoxically making sports compulsory for 14–16 year-olds.) The worry is that competitors elsewhere have been investing in excellent elementary and secondary education for decades past, and are now reaping the benefits. Will the newly discovered laggards have the stomach for the long haul ahead of them? □

Crick on consciousness

The London *Times* is in two minds on its sponsorship of a reductionist occasion.

THE opening sentence of J. D. Watson's book *The Double Helix* may be aptly paraphrased as "Nobody has ever known Francis Crick to write a dull book". For Crick's latest book is predictably far from dull (see R. L. Gregory, *Nature* **368**, 359; 1994). *The Astonishing Hypothesis*, subtitled "the scientific search for the soul" (Scribner's, New York, \$25.00; Simon & Schuster, London, £16.99), has inevitably caused a stir. In Britain, *The Times* has arranged with Dillons, the booksellers, that Crick should engage in a public debate at Westminster Central Hall, London, on 25 May. (Tickets cost £10 from The Times/Dillons Science Forum, Dillons, 82 Gower Street, London WC1 6EQ). But many must now wonder whether *The Times* is having second thoughts.

The book is about the relationship between mind and brain. Its essential message is that the collection of neurons with which the insides of our heads are endowed is an adaptive, 'self-organizing' system whose hard wiring is the product of evolution, but which is to some extent still malleable at birth. The machine is somehow able to make sense of the real world outside as well as of the inner world of personal consciousness. The precise attributes of the neuronal ensemble responsible for consciousness are not yet, of course, known. But that is more or less all there is to say about mind/soul and brain, although at present there is only a rudimentary understanding of what happens within the skull.

So the hypothesis is not particularly astonishing. Certainly it will not have astonished Crick himself. But there are many, to whom the hypothesis is at least mildly shocking. In a leading article on Monday (9 May), the newspaper predictably sets out to put some water between itself and the astonishing hypothesis. Consciousness is the stumbling-block. Both Descartes and Samuel Johnson are quoted as sources of the belief that conscious thought (not to mention soul) has no material correlates. The newspaper says that "only when the details are worked out can we be sure the reductionists are right" and, a little wistfully, that "proving it may turn out to be a long and difficult task". Many no doubt hope that the prediction is correct.

Consciousness is indeed the central issue, but Crick has himself gone a long way to describe the terms in which the concept can be discussed. The brain is not a kind of electronic computer, or something with analogues in artificial intelligence, but all these things and more, as well as being able to keep a retrievable record of its affairs. The immediate task is to enumerate those functions. But the adaptive value of cognition, especially in presenting those who practise it with a choice between alternative courses of action not necessarily dictated by the endocrine systems, is manifestly so great that the question is not whether consciousness can conceivably be the product of natural selection, but whether natural selection could have failed to single it out. The surprise is that neurons are such versatile cells. □

US makes 'boldest effort in years' to boost school science teaching

Washington. Nine cities were this week chosen by the National Science Foundation (NSF) to spearhead a bold attempt to overhaul the entire school science education system in the United States.

The so-called Urban Systemic Initiative (USI) is intended to address a situation which, American universities contend, leaves a typical 18-year-old high-school graduate 18 months behind his or her counterpart in Europe or Japan.

New York city — one of the chosen nine and the largest school district in the country — has also announced sweeping changes to the way science is taught in schools. These will eliminate general science courses, and will require students to pass advanced courses in mathematics and science before they can graduate.

The amount of new money involved in USI is not large, amounting to \$15 million over five years for each city. But the cooperative agreement that NSF will reach with recipient's will cover the spending of far larger amounts of the cities' resources.

"[NSF's contribution] is designed as a catalyst to enable the cities to put in place a new infrastructure for math and science education," says Luther Williams, assistant director for education at the NSF. He says the expenditure leveraged in this way will be 20 or more times the NSF's contribution.

USI is aimed at cities with many children in poverty. But with almost half of American children at school in urban areas, the initiative is also intended to improve overall standards.

The first USI agreements, due to be announced on 10 May, are with Baltimore, Chicago, Cincinnati, Miami, Dallas, Detroit, El Paso, New York and Phoenix. A further 16 eligible cities may join the scheme at a later date.

The initiative will trigger different action plans in each city, with the money going on a mixture of new curriculum development, teacher retraining in collaboration with local universities, school restructuring and other, sometimes experimental, reforms; Cincinnati, for example, will try out a year-round programme in pilot schools.

In New York, the schools chancellor, Ramon Cortines, announced last week that students will no longer have the option of taking non-academic mathematics and science courses to graduate. "The easy way out is the road to nowhere," Cortines pronounced, although a spokesman for the city's board of education was vague about where the extra resources would come from.

Jerrold Ross, of the school of education at New York University, says he welcomes any attempt to raise standards, but that questions remain. "Are the teachers prepared to

teach at this level?" he asks, pointing out that the city dropped its entrance requirements for teachers to get more of them into the classroom. "It's refreshing that someone is trying; but more money's going to have to come from some place."

In New York, the NSF's \$2-million contribution for the first year of USI represents \$2 for each child at school. But more money will come from the city, which has pledged to reform middle-school education to help students to cope with harder courses.

Although it is primarily a research funding agency, the NSF has been increasingly sucked into school education. Each year Congress — more concerned about the state of city schools than with funding scientists to write academic papers — diverts more of its money into programmes such as USI.

The NSF is already managing a large if diffuse Statewide Systemic Initiative (SSI) in 25 states and preparing a smaller Rural Systemic Initiative to help poor rural areas. It has already kicked one state — Rhode Island — off the SSI for failing to meet agreed benchmarks.

The initiatives also tie in with the Goals 2000 Educate America Act, which was passed by Congress in March and mandates the Department of Education to undertake similar programmes in non-science subjects.

But the federal government faces big problems in attempting to upgrade a schools system that is managed and financed at the local, county level, and where the money spent per student per year ranges from \$2,000 in parts of rural Mississippi to \$8,000 in Beverly Hills, California.

Shirley Malcom, director of education at the American Association for the Advancement of Science, and a member of the National Science Board which approved the USI grants, says the scale of the new initiatives makes them different: "We haven't tried to revamp the entire system before," she says.

But teachers' groups are sceptical. Bill Aldridge, director of the National Science Teachers' Association, says USI is well-focused, but many federal programmes lack substance. "They focus on administrative and structural changes, and completely neglect the fundamental problem, the lack of appropriate materials and poor preparation of teachers."

Aldridge is not sanguine about the prospects for reform. The New York plan reminds him of a Florida scheme some years back, which promised three years of science for every child. But without extra teachers and equipment, the scheme failed.

Colin Macilwain

This rearing, roaring cave bear (right) is just one of 250 specimens that will greet visitors to the Lila Acheson Wallace Wing of Mammals and their Extinct Relatives, which opens at the American Museum of Natural History (AMNH), New York, on 14 May. The opening coincides with the AMNH's 125th anniversary, and the refurbished gallery is the first of several that will reopen in a programme that extends through 1995 (dinosaurs) and 1996 (primitive vertebrates).

'Mammals' guides visitors through mammalian history using a cladistic rather than a chronological approach, in line with the museum's strong research commitment to fossil mammals. This accent on systematics is likely to cause a stir; when the Natural History Museum (NHM) in London tried the same trick a decade ago, researchers were outraged and visitors baffled by the result.

But any system is better than none at all. The NHM scrapped its own fossil mammal gallery (along with most of its research on fossil mammals) in favour of ecology, and the present hall — 'Discovering Mammals' — is best regarded as a



stopgap. In contrast, the NHM's new dinosaur hall, with its accent on biology rather than systematics, is an enormous success.

Henry Gee

Drug take-overs reflect changing industry

Washington. A spate of acquisitions, mergers and sell-offs last week illustrated how drug companies on both sides of the Atlantic are finding ways to survive cost-cutting and consolidation. At least for now, the trend is expected to continue.

Last July, Merck & Co. started the ball rolling by acquiring Medco Containment Services Inc., one of the largest drug wholesalers in the United States, for about US\$6 billion in stock and cash.

Since then, the shift in the purchasing, financing and delivery of health care in the United States towards more 'managed care' means that pharmaceutical benefit management companies (PBMs) such as Medco have gained in importance.

PBMs, which have become a powerful force in the delivery of health care over the past 5 years, manage insurance claims, ne-

gotiate discounts on drugs with manufacturers on behalf of payers such as large employers and health maintenance organizations, and deal in generic substitutes for brand-name drugs. An estimated 60 million Americans now fall under the control of PBMs.

Patricia Lea, an analyst with Vector Securities International, says that the growing importance of PBMs will "force others to integrate vertically or accept secondary positions in the pharmaceutical care game". With non-physicians taking on a more important and influential role in the decision-making process, Lea feels that in order to be major global players in the pharmaceutical industry 10 years from now companies will need to align themselves more closely with the likes of PBMs.

This strategy is now being emulated by the British company SmithKline Beecham

plc, which last week announced plans to acquire Diversified Pharmaceutical Services Inc., one of the four largest drug wholesalers in the United States.

The US company, a wholly owned subsidiary of United HealthCare Corporation, handles the pharmaceutical benefits of an estimated 11 million people. SmithKline Beecham is paying US\$2.3 billion for its purchase. As part of the deal, SmithKline has also forged a six-year alliance with United HealthCare. This agreement will provide SmithKline with the exclusive rights to data on 1.6 million members of health maintenance organizations owned by United, enabling it to make valuable comparisons on the cost-effectiveness of treatments.

This new alignment with a PBM is intended to enable the company to compete more effectively in the managed care arena. "Now is the time for us to move beyond the traditional role of selling pharmaceuticals toward a new role as a manager of total health care," said Jan Leschly, chief executive of SmithKline.

Roche Holding Ltd, which is based in Basle in Switzerland, also announced last week that it has made a \$5.3-billion bid to acquire the ailing Syntex Corporation, a health-care company registered in Panama with headquarters in Palo Alto, California.

The takeover is timely for Syntex. Recent sales figures have been sluggish, in part because the US patent on the company's main product, the non-steroidal anti-inflammatory drug Naprosyn (naproxen), expired last December.

Although Syntex has a dearth of new products for the near term, products in development for the treatment of pain and inflammation, including medicines to treat allergies, cardiovascular and cerebrovascular diseases, are expected to complement Roche's existing drug portfolio.

The deal, which will elevate Roche to fourth position in terms of worldwide sales, also makes sense from a logistical standpoint. Syntex is located near Genentech Inc., a leading US biotechnology company in which Roche acquired a major stake in 1990 for the sum of \$2.1 billion.

Eastman Kodak Co. has opted for the bold move of getting out of the drug business altogether. George Fisher, Kodak's recently elected chairman, president and chief executive, last week unveiled a new corporate strategy in which Kodak would divest itself of what he described as "non-core businesses". The move will allow Kodak to commit much-needed resources to its imaging businesses, where it is strongest. The company plans to sell off its pharmaceutical and consumer health products subsidiary, Sterling Winthrop Inc., and its clinical diagnostics division, but will retain its health sciences division. **Diane Gershon**

Microsoft founder backs gene sequencing

San Francisco. The founders of the most powerful US software company have joined forces with leading biotechnologists and gene sequencers to promote the activities of a small gene sequencing company in Seattle.

William Gates III and Paul Allen, founders of Microsoft Corporation, have invested \$10 million in Darwin Molecular Corporation, bringing the young company's total funding so far to \$14 million.

The investment is part of a larger financing which is being sought from the venture capital market and will be announced later this month.

Darwin was founded to develop small-molecule drugs using the technology of molecular evolution. This is a procedure that can potentially yield highly specific new drugs by subjecting one generation after another of targeted molecules to a survival test.

Darwin's original technologies were developed by Stuart Kauffman, one of the company's original founders, and its president. Kauffman still owns the original European patents, and US patent applications, although he has since left the company after a dispute with its current management, and now works as a professor at the Santa Fe Institute in New Mexico, as well as the University of Pennsylvania.

Over the past year, Darwin has linked its work on molecular evolution with DNA sequencing and sophisticated computer

analysis, in an effort to use information embedded in the human genome to identify potential targets for drugs against cancer, AIDS and autoimmune disease. It claims to have already created enzymes that may be useful in treating cancer.

Costa Sevastopoulos, a biotechnology venture investor who participated in earlier financing discussions with the company, says that Darwin was in a position to command a high price from Gates and Allen because of its expertise in molecular evolution and the names of scientists already associated with it.

They include Leroy Hood, a member of Darwin's scientific advisory board who played a key role in the founding of the Human Genome Project; Ronald Cape, chairman of the board and former chairman of biotechnology pioneer Cetus Corporation in Emeryville, California; and George Rathmann, chairman of ICOS Corporation in Seattle and chairman emeritus of the highly regarded Amgen Inc. based in Thousand Oaks, California.

Gates is said to have had talks with the company for more than a year before agreeing to invest in it. A biotechnology enthusiast, he has backed several young companies in the industry, including \$5 million in ICOS, \$5 million in Seattle-based Targeted Genetics and \$7.5 million in Ligand Pharmaceuticals Inc. in San Diego. Gates also contributed \$12 million to the University of Washington to lure Hood from the California Institute of Technology to Seattle.

Allen, Gates and Rathmann are to join Darwin's board. Gates and Allen will apply their knowledge of technology and information analysis, as well as their business acumen, to Darwin's medical objectives.

Sally Lehman

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**Gates: survival of
the fittest?**

Box Features

Uranium fuel sparks German-US controversy

Munich. More than 20 of Germany's top physicists have sent a letter to ministries, politicians and licensing authorities in Germany expressing concern over the proposed use of highly enriched uranium (HEU) in a new research reactor planned for construction in Garching near Munich.

Their main complaint is that the so-called *Forschungsreaktor München II* (FRM-II) would as currently planned undermine attempts led by the United States to eliminate the world-wide use of HEU in research reactors, and to substitute it with the less energy efficient but safer low enriched uranium (LEU).

The United States, at present the west's only supplier of HEU, has introduced strict controls on the distribution and use of this fuel, quoting its commitments under the terms of the Nuclear Non-Proliferation Treaty (NPT), which came into effect in 1970. In addition, over 50,000 individuals in Germany, including many scientists, have backed a demand that the FRM-II be redesigned to use LEU fuel.

But the scientists at Munich's Technical University who have designed the FRM-II argue that converting it from HEU to LEU would be extremely costly. They also claim that such a move is unnecessary, as Germany is a signatory of the NPT, and thus has strict controls on the use of nuclear fuels.

Last week saw the opening of an inquiry into the planned reactor, which will provide high energy neutrons for researchers in materials and medical sciences. German physicists have been trying to establish a new national neutron source since the late 1970s, as the country's four working research reactors are ageing, and have neutron fluxes too low to meet all current research needs.

Planned for construction next to Munich university's existing research reactor, known as the Atom-Ei (atomic egg) because of its shape, the new reactor would have a high neutron flux (800×10^{12} per second per cm^2) and would cost DM525 million, two thirds paid by the federal government, and the rest by the state of Bavaria.

Wolfgang Gläser, professor of experimental physics in Munich and former director of Europe's most powerful research reactor at the Institut Laue-Langevin in Grenoble, France, says that the use of HEU, made up of 93 per cent ^{235}U and 7 per cent ^{238}U , is needed to achieve the required neutron flux at a power of 20 megawatts.

If the new reactor is required to use a mixture of only 20 per cent ^{235}U (and 80 per cent ^{238}U), he says, it would have to operate at twice this power, raising annual running costs from DM20 million to DM30 million. In addition, conversion is likely to cost an estimated DM200 million.

Gläser also argues that LEU provides a

similar security risk to HEU, as ^{238}U in the fuel is converted to plutonium. But Werner Buckel, former president of the German Physics Society, says that sophisticated reprocessing technology is required to extract this plutonium, which is already at low levels, and that the risks are therefore not comparable.

The United States has established a programme to develop alternative high density LEU fuels. Its overall policy, intended to reduce the risks of nuclear proliferation, was reinforced by the Schumer amendment to the 1992 Energy Policy Act, which specifies three conditions for the supply of HEU



Gläser: claims fuel would be safe.

to research reactors.

First, the reactor must be technically incapable of using any of the LEU fuels currently available. Second, the relevant national government must agree to use an alternative, compatible LEU fuel type, if one becomes available. Finally, the United States must become involved in developing an LEU fuel type that would be compatible with the specified reactor.

Despite the extra costs incurred by reactors using LEU fuel, the policy has so far been highly successful. Thirty eight of the 42 research reactors outside the US which depend on imported US fuel have already switched, or are preparing to switch, to LEU. These include Germany's four current research reactors in Berlin, Hamburg, Jülich, and the Atom-Ei in Garching. One of the remaining four is now considering switching, and the other three are not technically capable of conversion.

Given this virtually universal compliance with the policy, as well as Germany's ultra-sensitivity to 'green' issues, the country's insistence on using HEU at Garching has generated widespread surprise.

Government officials deny that the use of HEU will increase the risk of nuclear proliferation. They point out that strong security measures have been incorporated into the FRM-II plans to meet the demands of both the European Atomic Energy Community (Euratom) and the International Atomic Energy Agency.

But Robin Delabarre from the US State Department's section on nuclear affairs says that this is not the point. "The German safeguards are fine," he says. "But it is not a problem specific to Germany; there is a general concern about the risks of international transport and use of weapons-grade materials."

The US is particularly worried that, by breaking ranks, Germany could encourage those responsible for research reactors in other countries to reconvert their reactors to use the cheaper HEU fuel. If that happened, however, a new question would arise concerning the origins of the fuel.

Gläser says he is confident that the US will agree to supply FRM-II with HEU, accepting the reactor as an exception to its general rules on the grounds that a redesign to use LEU would be uneconomic. But Delabarre says that economic reasons are not sufficient to allow an exception, and that a request for HEU from Garching would "most likely not be approved".

The State Department has been urging the Garching team — so far unsuccessfully — to work with US scientists at the Argonne National Laboratory near Chicago on low enriched fuel that would be both technically and economically acceptable.

If the US refuses to supply the HEU (no such fuel has been exported from the US since 1992) and the reactor is not converted to use LEU, its fuel will have to be sought elsewhere. It will have to be ordered through Euratom, as nuclear installations in Germany, as in all other countries of the European Union, are obliged to do.

A spokesperson for Euratom admits that US policy has put its HEU supplies "in grave doubt in the near future". The organization is considering new sources — possibilities include the United Kingdom, France, and Russia — but will not discuss the options it is considering.

The public hearing, which is part of the nuclear licence procedure for FRM-II, is likely to continue for several weeks. Bavaria's prime minister Edmund Stoiber says he would like to see a (positive) licensing decision taken before the state elections in September. But few expect a decision much before Christmas.

Alison Abbott

Business booms for China's universities

Beijing. Entrepreneurialism seems to be thriving in China's universities. According to a report from the Xinhua news agency in Beijing, the output of university-run enterprises in Beijing reached a value of 2.6bn yuan (US\$330 million) in 1993, more than twice the figure for 1992. Total profits also doubled, to a total of 400m yuan.

Of Beijing's 67 colleges and universities, Beijing University, Qinghua University, the Beijing Polytechnic University and the Beijing University of Science and Engineering, each reported an annual output of more than 100m yuan from their campus-run hi-tech enterprises. □

Losers could still be winners in ESA space competition

Paris. The Space Science Advisory Committee of the European Space Agency (ESA) last week selected four out of seven space science proposals in the semifinal of a competition that will eventually lead to the selection of one as a 'medium-sized' science mission to be sent into space in 2003.

But, spoilt for choice, ESA did not reject the remaining proposals. Two will be considered for upgrading to 'cornerstone' missions, and the other will be reviewed internally to see if it can be combined with hardware from other planned missions. The advisory committee's recommendations will probably be endorsed by ESA's Science Program Committee in June.

The four finalists are:

- **MORO**, a lunar orbiting observatory aimed at studying the Moon's surface and interior;
- **INTERMARSNET**, a network of 7.5-kg stations to be sent to the surface of Mars to study its atmosphere, surface and internal structure;
- **COBRAS/SAMBA**, a satellite to measure cosmic background radiation, which its promoters claim has ten times more sensitivity and 50 times greater resolution than NASA's COBE satellite; and
- **STEP**, Satellite Test of the Equivalence Principle, one of last year's finalists, which will carry out several experiments in basic physics.

ESA will furnish each of the four finalists with ECU800,000 (US\$900,000) to finish a further year's study, including industrial development. The final winner will emerge following a similar competition next year. ESA will budget ECU345 million to build, launch and operate what will be its third medium-sized mission. The losers can run again in future years.

ESA also decided to refer the STARS proposal for a stellar physics mission to an internal study, without industrial involvement, to see whether its cost could be reduced by using a 'bus' developed for either the XMM or Rosetta mission.

The advisory committee also proposed upgrading two of the seven proposed projects — LISA and Mercury Orbiter — to 'cornerstone' missions. The budget for the latter missions is around double that of medium missions, and better suited to both proposals, says ESA. "They were very good, but very expensive."

LISA (Laser Interferometer Space Antenna) would search for gravitational waves from black holes, neutron stars, white dwarfs, and the Big Bang. The Mercury Orbiter satellite would have arrived at Mercury in 2008 and studied the planet's geology and surface composition. **Declan Butler**

CSIRO loses out in switch to 'strategic' research

Sydney. Paul Keating, the Australian prime minister, announced last week that his government intends to spend A\$60 million (US\$84 million) on creating seven new strategic research centres, and another A\$118 million on a string of 'technology diffusion' centres across the country.

At the same time, however, the government plans to cut A\$20 million from the budget of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), the country's largest research organization, which last year was A\$670 million. The cut is likely to result in the loss of between 200 and 300 staff.

Ironically, the decisions were announced to parliament as part of a white paper (policy document) on reducing unemployment. Most of Keating's statement focused on detailed proposals for doing this. But it also contained various science policy initiatives, including a proposal for an eight-year programme to support "major national research facilities".

Few details have been released about how the programme would work. But a spokesman for Peter Cook, the recently appointed minister for science, said the money would be used only to set up the centres.

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Keating: aiming to cut unemployment.

Operating costs would have to be found by the centres themselves, or by the institution or department responsible for them.

Cook said his department had a list of proposals including a number from other departments. Outside proposals would also be accepted. The most advanced proposal is for a Nanotechnology Facility. Keating said the facility would build on the work of the Cooperative Research Centre for Molecular Engineering — recently created under a separate government programme — which was developing nanotechnology devices for the health-care, food and environment industries in Australia.

The second major initiative, the technology diffusion centres, will involve setting up demonstration sites of new technology so that business owners and executives can see the technology in action. The spokesman for Cook said the sites would help overcome the reluctance of businessmen to adopt new technology.

The CSIRO has been given an extra A\$10 million to help it work more closely with small and medium-sized enterprises, as well as A\$7 million to improve business access to advanced technologies. But it will suffer a \$20 million cut in its annual budget for the next three years.

Sources in CSIRO said the jobs lost would be initially in the administrative and support areas, and that several divisions (the basic operating units) would be cut into smaller research centres.

Mark Lawson

French academy baffled by language rule

Paris. The French Academy of Sciences last week accused the government of being out of touch with the research community, after the National Assembly had ignored repeated requests to exempt international scientific conferences from provisions of a bill on the use of the French language (see *Nature* 367, 304; 1994).

Last month, the academy published a public appeal, entitled "French science is in danger!", asking the government to exempt scientific congresses attended by a majority of non-French speakers from the requirement that French can be spoken. The bill, which was adopted by the National Assembly last week, also requires all written contributions (and the subsequent proceedings) of such conferences to include at least summaries in French.

Paul Germain, the permanent secretary of the academy, says that the bill would make it impossible for French organizations to hold international con-

ferences in France. He says that it would be impractical to produce proceedings in French, and financial suicide to have to supply simultaneous translation.

But, in a further blow to the academy's campaign, the National Assembly also adopted an amendment requiring all research supported by public funds to be published in French. Germain says he is baffled by what this is intended to mean in practice, but adds that he is shocked that the government, despite its "good intentions", has "absolutely no idea" of how the scientific community works.

The academy says it will continue to fight the bill during its second reading in the Senate. The Centre National de la Recherche Scientifique for its part says that the defence of the French language depends mainly on having the quality of research and funds needed to attract foreign researchers — and their families — to France.

Declan Butler

NIH scientists pressed to maintain standards

Washington. The scientific directors of the 24 institutes that make up the US National Institutes of Health (NIH) will lose some of their powers of patronage and face tough performance monitoring under reforms being backed by both Harold Varmus, the director of NIH, and by congressional decision-makers.

The reforms have been proposed by an independent high-powered panel set up by Congress. In a report released last week, the panel says that the \$1.3 billion in-house research programme, based at the main NIH campus at Bethesda, Maryland, is too fragmented, and faces a mediocre future unless steps are taken to revamp it.

The report proposes new structures to monitor and control the quality of the intramural programme of each of the institutes and centres that make up NIH, as well as a new board that would vet all appointments of tenured research staff.

It also contains proposals for the step-by-step renewal of the Clinical Center, the unique but dilapidated 450-bed research hospital at the heart of the Bethesda campus. These include the construction of a new 250-bed hospital and the renovation of existing laboratories in the centre.

Varmus plans to use the report as ammunition in his drive to impose more consistent standards across NIH, which the report describes as "balkanized". He also wants the NIH intramural programme to become a supreme centre of excellence at which the best young biomedical researchers will aspire to work.

But this is a tall order, given the financial resources of some of the universities and private institutions competing for the same people. Varmus's room for manoeuvre is also restricted by the high degree of autonomy the institutes enjoy.

The congressional subcommittee in charge of funding NIH asked for the report last autumn, chiefly because of its concern about the cost of renewing the Clinical Center: estimates have ranged from \$900 million for repair to \$1.6 billion for full reconstruction.

The panel, co-chaired by Gail Cassell of the University of Alabama at Birmingham and Paul Marks of the Memorial Sloan-Kettering Cancer Center, New York, found that the balkanization of the intramural programme "has contributed to unevenness in quality, quality control and productivity" at NIH.

It recommends that the institutes appoint more genuinely independent scientists to their boards of scientific counsellors, and that these boards in turn do more to keep an eye on the scientific directors, whose alleged omnipotence has been a focus of criticism at NIH.

The institutes are also asked to construct an annual planning process to divide resources sensibly between the intramural programme and the extramural one, which consumes the bulk of NIH's \$11-billion budget. Anthony Fauci's National Institute for Allergy and Infectious Diseases has a process which, the report suggests, the others should use as a model.

Another recommendation is that all tenure appointments be vetted by a centrally appointed board of NIH scientists serving staggered terms, instead of by the board of scientific directors as at present. Mike Gottesman, acting deputy NIH director in charge of the intramural programme, who would appoint this new board, denies that the change would centralize power. "It'll be a more diverse group of people, somewhat more separate from the administrative side of the programme," he says.

The panel says that the intramural programme's share of the NIH budget should not grow above its present 11.3 per cent, and that weaker elements should be cut back to fund renewal of the Clinical Center. But it did not question the fundamental need for a large intramural programme, as some critics have done. "A strong intramural programme is essential for a strong extramural programme," says Marks.

NIH will spend \$2.5 million over the next year drawing up more detailed plans for the renewal of the Clinical Center. These will probably involve the refurbishment of parts of the existing building, the demolition of other parts and the construction of the new hospital as an annex.

Many of the proposed reforms have been suggested before, but Marks says he is optimistic that this set will be carried through because of support from Varmus, the Health Secretary, Donna Shalala, and the key staff members on con-



Where next? NIH's Clinical Center.

gressional committees.

Some statistical evidence supports the popular perception that standards have declined at NIH over the past decade (see chart below). But its defenders point out that the proliferation of centres of excellence in biomedical research elsewhere in the United States makes a relative decline almost inevitable. Varmus says he is as confident as ever that the intramural programme can be reinvigorated.

Colin Macilwain

Postgraduate plans under fire in UK

London. Britain's Royal Society has rejected as "educationally unnecessary" a proposal by the Office of Science and Technology (OST) to require all those wishing to pursue a PhD to undergo an initial year of training, leading to a new MRes qualification.

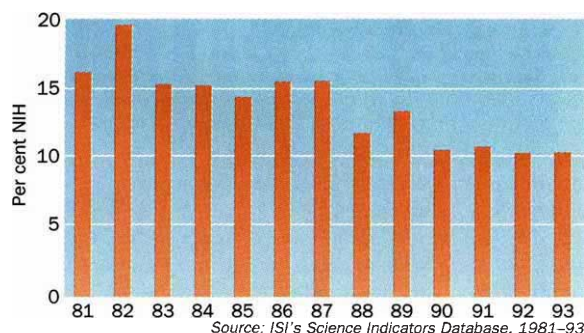
The OST's proposal was one of the key recommendations in last year's white paper on science. This announced the government's intention to ensure that a master's year should become "the normal initial postgraduate qualification for research students supported by the research councils."

Detailed proposals of how this scheme might work were published in a consultation document last year by the OST. But in its reply, published last week, the Royal Society echoes the feelings of many professional scientific bodies that the goals being pursued by the government could equally well be achieved by other means, such as the gradual introduction of four-year undergraduate courses.

"We are not against the MRes in principle, but feel it is not the only solution to the problems that the government has identified," says one individual involved in preparing the society's response.

Not all responses to the government's proposals have been as critical. According to the Confederation of British Industry, there has been a positive response from some engineering employers, while the new Engineering and Physical Sciences Research Council is believed to be enthusiastic. □

Percentage of NIH papers ranked within ~ 300 most cited articles published each year



Research centre head quits in frustration at UK policy

London. Britain's efforts to boost the integration of basic and clinical research in gene therapy received a major setback last week with the resignation of Kay Davies, one of the country's leading geneticists, as director of the Medical Research Council's (MRC's) new Clinical Sciences Centre (CSC).

Davies was appointed in 1992 to head the centre, which is due to open later this year at the Royal Postgraduate Medical School, located at the Hammersmith Hospital in west London. Her experience in linking basic and clinical studies of various diseases, and in particular her work on the genetic basis of muscular dystrophy, made her an ideal appointment.

Since then, however, planning for the centre has become bogged down in political fighting over the government's reorganization of London hospitals, a move intended to address a combination of declining patient demand in the capital and the placing of health care on a 'purchaser/provider' basis.

Last month, the government approved plans to merge the Hammersmith Hospital and nearby Charing Cross Hospital. But it refused to supply the additional funding which, it had been told by leading scientific advisers, was needed in order to protect research programmes in any merger of the two institutions on a single site (see *Nature* 386, 177; 1994).

The government's decision seems to have been the last straw for Davies, who had spent the previous two years trying to recruit scientists to a centre whose future is now uncertain, and fighting unsuccessfully to persuade the government to provide for its long-term security.

"My decision to resign was based entirely on the fact that I had put two years of my life into fighting for something I have always believed in, namely linking basic research to its possible clinical applications," Davies said last week. "The CSC was a golden opportunity to do just that; now I just want to get on with my research."

Sir Dai Rees, secretary of the MRC, which has invested £11 million in the CSC — and had been hoping to persuade the government to subsidize a transfer to the Charing Cross Hospital — has expressed his "great regret" at Davies' decision. But he added that the opening of the CSC will proceed as planned, and that a search for a new director will begin shortly.

Nevertheless, the council faces an uphill task in restoring the credibility of a project that has been severely damaged — both practically and psychologically — by the government's decision on the Hammersmith/Charing Cross merger and, now, by Davies' departure.

Those anxious about the future include a number of research groups from the MRC's Clinical Research Centre (CRC) at Northwick Park, which are due to move to Hammersmith when the CRC closes down later this year. "It has certainly had a demoralizing effect," says one scientist, claiming that various colleges of the University of London are now keen to offer the CSC (or at least some of its component parts) an alternative home to the Hammersmith.

"The impression that is left is of a country working without an endocrine system; no-one seems to know what anyone else is doing," he says. "Given the amount of work that went into preparing the Hammersmith proposal, it is going to be very difficult if things start to fall through at this stage."

There is also gloom at the Wellcome



Davies: returning to the laboratory.

Trust, which had already committed £4 million towards research facilities adjacent to the CSC laboratories in a new clinical research building at the hospital, and had agreed to support five research groups in the laboratories.

Bridget Ogilvie, the director of the trust, said last week that she was "sad, but not surprised" at Davies' decision, which she described as a "major blow" to the CSC. Given the uncertainties over the future of the Hammersmith, she said, the trustees are meeting soon to reconsider the trust's position. "We are still discussing what our options are," said Ogilvie.

Davies is reluctant to talk of her own plans, beyond the fact that in the short term she intends to devote her efforts to the work of her research group at the Institute of Molecular Medicine at Oxford. But she is rumoured to be a strong candidate for the position of professor of genetics at the University of Oxford, which could be combined with responsibility for some of the genetics work carried out at the MRC's Radiobiology Unit at Harwell.

Ironically, Davies chaired an expert working group that recently reported on the state of genome research in the United Kingdom (see *Nature* 368, 675; 1994). The report argued the need for Britain to exploit both its scientific strengths in human genetics and the opportunities offered by its clinical base through the National Health Service. The events surrounding the CSC indicate that, in the current political climate, achieving synergy between the two is considerably easier said than done.

David Dickson

Rhône-Poulenc to focus biotech work on gene therapy

Paris. Gene therapy in France has taken a big step from the laboratory bench to the hospital bed with the decision by Rhône-Poulenc-Rorer (RPR), the pharmaceutical subsidiary of the Rhône-Poulenc group, to concentrate its biotechnology business on cancer gene therapy.

To do this, RPR has set up a new division, known as Biotech. This regroups RPR's biotechnology activities, including the 250 staff at its biotechnology centre at Vitry in the south of Paris, and its 37 per cent stake in the US start-up gene therapy company Applied Immune Sciences.

Moreover, in a novel approach to the organization of drug research in large companies, RPR has offered the new division the freedom of an independent biotechnology company. It may eventually be floated on the stock exchange.

RPR had tentatively explored gene therapy through collaborations with academic research teams. "We've now decided that we can do it [commercially]," says Jean-Bernard Le-Pecq, scientific director of Biotech.

One reason for plunging head first into gene therapy, admits Le-Pecq, is that RPR missed the boat in the development of recombinant drugs, and watched while its competitors captured all the obvious candidates, such as growth hormone. "It was an interesting page of pharmaceutical history, but written by others," he says.

But Le-Pecq says that setting up an industrial strategy for gene therapy poses an unprecedented problem, namely that the intellectual property rights to gene therapy techniques are held by academic groups and biotechnology companies. "It's like trying to build a railway when different people own the trains, the rails, and the signals."

RPR, he says, is at present involved in persuading patent holders to pool their intellectual property rights in order to speed up the transfer of gene therapy from the laboratory to the factory. A network similar to that of the software company Microsoft is the most probable outcome. One natural node will be the Généthon laboratory in Paris, which has launched two large gene therapy programmes (see *Nature* 365, 686; 1993).

RPR's parent company, Rhône-Poulenc, is itself shortly to launch a major programme in chemistry corresponding to the five-year, FF1,600-million programme launched jointly with the government in 1991 in the life sciences.

Chimie-Avenir, as it will be called, will probably involve not only Rhône-Poulenc but also other industrial groups such as Lafarge-Coppee, Saint-Gobain and Bouygues.

Declan Butler

Environmental agency responds to its critics

Washington. After coming under fire for lack of rigour and focus in its research programmes, the US Environmental Protection Agency (EPA) is carrying out a top-to-bottom review of the way it conducts and evaluates research.

Several internal studies will assess virtually every aspect of EPA science, from the fate of the agency's laboratories to its policies on peer-review and outside scientific advice. The aim is to reinforce the claim of its director, Carol Browner, that "science is the backbone of everything we do".

In recent years, the agency's critics have included Congress, independent study groups and even its own administrators. A particular target has been its activities in critical areas such as risk assessment.

To improve its image, Browner has taken a number of steps, including the creation of a Science Policy Council of senior agency managers to coordinate research activities scattered among half-a-dozen different programme offices.

One priority for the council is to standardize the rules governing outside peer-review of EPA research before publication. The new rules, which are expected to be in place by September, will be sufficiently flexible to allow different levels of review depending on the type of publication, Sylvia Lowrance, an associate deputy administrator told the agency's Science Advisory Board (SAB) last month.

At the request of Congress, the agency has also commissioned the Mitre Corporation to carry out a study of its field laboratories. There are 12 environmental research laboratories under the direction of the Office of Research and Development (ORD), and about 30 smaller facilities, whose missions range from technical support of programme offices to vehicle emission testing.

Mitre's report will be presented to EPA at the end of this month, and a decision on the fate of the laboratories is expected in early June. The options range from leaving them as they are to consolidating them into four 'mega-laboratories' based on their missions, as recommended in a 1992 Carnegie Commission study.

But even if Congress does allow EPA to close down major laboratories in an election year — which itself is doubtful — many observers say this kind of deck-shuffling misses the point of what is really wrong with the way EPA research is structured.

Terry Yosie, a former SAB chairman, calls efforts to consolidate the laboratories "very ill-conceived", and says that the agency has always preferred to focus on "fixing" them rather than addressing other issues, such as the top-heavy management at ORD headquarters, an issue which the SAB has raised repeatedly.

His criticism is echoed by a current mem-

ber of the board, who says that EPA "needs to step back and ask what do all those people do in Washington". But such remarks point to another problem, namely that the agency has tended to ignore the advice of its own science advisory board.

Perhaps the most public of the many problems plaguing EPA's scientific efforts has been the struggle to attract a prominent scientist to lead its office of research. That may soon be resolved by the expected appointment of Robert Huggett of the Virginia Institute of Marine Sciences.

But critics claim that, without a clear mandate from the top for long-term basic research, as well as consistent funding, EPA

will remain a regulatory agency at heart, with little aptitude for scientific work.

At last month's SAB meeting, the members of the executive committee expressed disappointment that research was not given greater weight in the Clinton administration's proposed 1995 budget.

Roger McLellan of the Chemical Industry Institute of Toxicology, who chairs the board's Research Strategies Advisory Committee, said the 1995 budget continued a pattern of "constant erosion" of both dollars and people for ORD since the 1980s. In all that time, he said, "we haven't put anything into the science base."

Tony Reichhardt

MRC to fund mouse genome centre?

London. Britain's Medical Research Council (MRC) is due to give its verdict within the next few months on an application to create at Hinxton Hall near Cambridge what could eventually become a European centre dedicated to the genetic and physical mapping of the mouse genome.

Last month, a group of leading British geneticists warned in a report commissioned by the Office of Science and Technology (OST) that more support for mouse genetics was needed to prevent the country from losing ground against major US research programmes.

No final decision has been taken either on funding the mouse genome centre or on where it would be built. But facilities owned by the Wellcome Trust at Hinxton Hall, Cambridge — already the site of the Sanger Centre and the European Bioinformatics Institute, with the MRC Human Genome Mapping Project Resource Centre arriving in June — would, say MRC officials, be "a very attractive possibility".

According to Stephen Brown of St Mary's Hospital Medical School in London, a key figure in the submission to the MRC, the close similarity between the genomic sequences of mice and humans means that mouse models can provide important help in cloning human genes.

Brown has been closely involved in a joint project between the Institut Pasteur in Paris and the MRC Resource Centre to create the European backcross, a family of 1,000 mice obtained by crossing two different species. The backcross has rapidly gained an international reputation as its sheer size allows genetic mapping at a much higher resolution than any other family of mice so far created.

According to the OST report, however, there is now a danger that groups with greater resources — such as Eric

Lander's team at the Whitehead Institute in Cambridge, Massachusetts — could reap the fruits of Europe's labour.

Brown says the proposal would neither compete with Lander's work nor duplicate it, and that their approaches to mapping are "entirely complementary". Indeed, the Whitehead Institute has recently started a collaborative programme with Britain to put 6,000 genetic markers on the European backcross map, a preliminary to the physical mapping — and eventual positional cloning — of genes.

But Lander has already placed 4,200 markers on his own genetic map stemming from a much smaller family of mice, although with much coarser results than the European backcross could offer. He intends to pepper the genome with a total of 6,000 markers by the end of the year — long before the British effort is likely to be up to speed.

Some supporters of the British mouse genome centre argue that the need facing Britain is not to catch up with the United States but to consolidate its present lead.

But others argue that, at least in the near future, Lander is likely to play the leading role in sequencing the mouse genome, and that the need facing Europe is "to decide how it can be a real partner, rather than having to pick up scraps off the floor".

Even if only battling for second place, the new centre, surrounded by a community of genome workers at Hinxton Hall, could develop into the hub of mouse genetics research in Europe. Brown plays down such ambitions. But others say that a British centre in such a location stands a good chance of eventually becoming a European centre, given in particular the potential for comparative studies of the human genome offered by the proximity of the Sanger Centre. **James Younger**

Close contact and AIDS

SIR — Max Perutz¹ discusses the question you raised of what a public official (or anyone for that matter) should do when he knows that official policy poses potential dangers. This problem arose in the case of Professor Jean-Pierre Allain, jailed last year in France for failing to prevent the distribution of HIV-contaminated blood-clotting factors to haemophiliacs. Perutz mentioned that Allain, alarmed by the use of HIV-contaminated blood, attempted to voice his fears to the public by giving an interview to the French newspaper *Le Matin*, but the interview was not published. This made me wonder what lesson might be drawn from the difficulties I had in publishing critical remarks about statements released in 1986 by the US Surgeon General and by the Institute of Medicine of the National Academy of Sciences concerning the risks of HIV transmission.

The surgeon general² said about family members living with HIV-infected individuals that: "There is no evidence of transmission (spread) of AIDS virus by everyday contact even though these family members shared food, towels, cups, razors, even toothbrushes, and kissed each other." The report of the Institute of Medicine³ similarly stated that "there is now substantial evidence against transmission through so-called casual contact, including regular close contact (such as that occurring in sharing accommodations, eating utensils, or even toothbrushes) . . .".

Both statements were based primarily on one scientific paper, by Friedland *et al.*⁴. That paper was accompanied by a guest editorial⁵, which concluded: "The evidence presented by Friedland *et al.* is a powerful argument to counter the public's fear of casual contagion . . .". Unfortunately, careful reading of the Friedland paper revealed that their study was quite limited, and this was soon pointed out⁶; the study could not rule out a risk as high as one chance in about 35. Yet such a crude study appeared to provide the stated foundation for government policy.

My contribution was to do a survey among scientists to find the largest risk of household transmission of HIV that they associated with the comforting words "extremely and reassuringly low" subsequently used by Friedland *et al.*⁷; in brief, the survey indicated a risk of roughly 1 in 100,000. My aim was to point out the great disparity between what officials claimed was proven and what was actually proven, but neither *Nature* (in 1987) nor *Science* (in 1989) would publish it. The *Lancet*, to its credit, published the essential elements of my letter virtually upon receipt⁸.

I do not know what current studies show about the risk of HIV transmission by household contact. I do know, however,

that in 1986 there was a vigorously stated claim that the risk was negligible, but the evidence cited to support the claim was woefully inadequate — by a factor of about 3,000. Although a deficient scientific evaluation was uncritically accepted by a large section of the scientific community and was cited widely and approvingly in public forums, the two leading general science journals declined to air the matter. I think there may be a parallel with the failure of *Le Matin* to publish the interview with Allain.

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Ponzi economics

SIR — *Nature* confirms (368, 177; 1994) what many working academic scientists have long suspected; namely that during recent years, US universities mortgaged the indirect-cost components of future grants to build new facilities on the speculation that these facilities would attract sufficient new grant funding to pay for the building spree. Outside the groves of academe, pyramid schemes of this kind are generally named after the celebrated swindler Charles Ponzi, whose operation collapsed in the early 1920s.

The panic among the administrators of the American Association of Universities is due to the approaching collapse, belatedly visible to them, of their own Ponzi operations. It is no surprise that they wish the cost of the collapse to be shifted to the research enterprise as a whole, and away from the indirect-cost charges they have been using for leverage. This simply reveals their true inclinations, as does their view that university success is measured not by the quality of the research or the character of the education, but rather by the ability to "expand aggressively" (as Dennis Dougherty of the University of Southern California so nicely puts it) by means of Ponzi economics.

Although pyramid schemes are not unheard of in normal commerce, they are subject to prosecution when false representations are made. One wonders what representations university officials made to the National Institutes of Health and

the National Science Foundation in order to justify their indirect-cost charges. Did they reveal that these funds were used to leverage financing for new facilities in the hope of adding still more grants (with still more indirect-cost return) to the pyramid? An investigation of this subject might result in some of the most inventive university officials joining counterparts in the investment business, such as Charles Keating and Michael Milken, in a period of residence at another kind of federal institution.

This outcome might wonderfully concentrate the minds of academic vice-presidents on those functions that distinguish a university from an acquisition holding company. Moreover, an improvement in the climate of integrity in the academic world would improve the environment for honest research and serious education, two functions that may still have a place at research universities.

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Slovak science

SIR — Alison Abbott's pessimistic article, "Slovak science lacks finance, direction . . ." (*Nature* **366**, 386; 1994) is still too optimistic.

The total budget of the Slovak Academy of Sciences, which was between SK800 and SK900 million a year in the late 1980s, has fallen to SK381 million this year. And inflation during this five-year period is close to a factor of three, so that the budget has decreased in real terms by a factor of more than five.

But conditions for doing science worsened considerably in the late 1980s. Salaries were then close to a half of the science budget, but they are now a much higher proportion. This has not resulted in an improvement in the standard of living of research scientists. Some junior scientists with PhDs who are married with children are earning less than what is recognized as a living wage. After subtracting salaries from the science budget, the shrinkage of the remainder is much more evident. Energy and mailing costs are rising more quickly than inflation, so the money available for library facilities, instruments, equipment, travel and so on has decreased considerably.

The quality and amount of science in Slovakia has not fallen, but the continued existence of basic research is in question. Nevertheless, the quality and amount of science has not fallen — at least at this institute.

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AIDS: time to turn to basic science

Bernard N. Fields

Success in controlling the AIDS epidemic is as likely to arise from unrelated areas of research as from AIDS-directed programmes.

IN the 1980s, it became clear that AIDS was a new disease and a devastating epidemic. In an attempt to understand and control it, governments in the United States and elsewhere supported research that emphasized basic and clinical investigations on the human immunodeficiency virus (HIV), the disease AIDS, and its medical sequelae. In many aspects this research programme has been highly successful. Drugs have been developed to extend the lives of people with AIDS, and much attention has been paid to dealing with manifestations of the disease, such as Kaposi's sarcoma. But the ultimate goals of preventing and effectively treating this epidemic infection have not been achieved.

It has been approximately 10 years since the discovery of the virus that causes AIDS. Enough time has passed to assess the approaches taken in attempting to cure the disease. It is now imperative to ask whether the approaches adopted in the early years of AIDS research are the ideal ones for the next decade. The fact that a large bureaucracy and infrastructure have become established during these early years may make it difficult to implement a real change. But I think that we need to alter substantially the way we set priorities and fund AIDS research.

I acknowledge the difficulties of changing the status quo, as well as the inherent uncertainties of any predictions of the future, but it is my view that we need to bring fresh scientific thought to the problem of AIDS. We need change, but what kind? We need to put increased emphasis on basic science and broaden our definition of AIDS-related research. In addition, we need to look critically at the time, effort and money spent on many aspects of drug development and vaccine programmes that have little or no scientific rationale, and are thus unlikely to lead to effective therapies or prevention. The focus on drugs and vaccines made sense a decade ago, but it is time to acknowledge that our best hunches have not paid off and are not likely to do so.

Gaps in our knowledge

The real goal of policies for funding AIDS research should continue to be to hasten the solution of this complex scientific, medical and human problem. If the money allocated to AIDS research is to be spent wisely, finding effective therapeutic and preventive measures must be based

on adequate scientific and epidemiological information.

First, let me emphasize that the first decade of AIDS research was productive, resulting in an understanding of many aspects of a totally new disease. This was due, in large part, to the technologies and knowledge base available at the time. Thus, within a short time, HIV, a new retrovirus, was recognized as the principal cause of AIDS; effective diagnostic tests were developed; and HIV was studied at the molecular level and its unique gene organization and complex mechanisms for replication were uncovered. In addition, a great deal was learned about AIDS as a disease and its epidemiology. This explosion of information led to a certain degree of optimism about the potential for treating and/or controlling AIDS. The rapid development of drugs (such as AZT) directed at the reverse transcriptase enzyme in HIV seemed to presage real progress in developing effective treatment.

But things turned out to be less simple. AZT, for example, certainly has a role in the treatment of patients with HIV-1 infection, especially in reducing transmission of virus from HIV-1-seropositive mothers to their newborn, and in treating patients with advanced disease. But the first results of the Concorde study are a reminder that AZT has limited value when given to patients who do not have advanced disease, and that it is not a very effective drug.

Despite rapid progress in certain areas, serious limitations in our capacity to study HIV infection have become glaringly apparent. The initial lack of an animal model meant that fundamental questions about the pathogenesis of HIV infection and AIDS could not be addressed. Understanding the pathogenesis of AIDS is central to an appreciation of the way in which HIV weakens the immune system and how the virus could be modified to stimulate effective immunity in infected men and women. The lack of an animal model was solved, in part, when infection by the simian immunodeficiency virus (SIV) in monkeys was recognized as a natural counterpart to the human infection. Other animal models have appeared, but there is no truly useful small-animal model that faithfully replicates the human disease. (Unfortunately, the SCID-Hu mouse is useful only for very limited applications.) In fact the SIV model has

begun to provide what may be the most compelling evidence that a vaccine has a chance of working — although the most effective SIV candidate so far, a live-attenuated deletion mutant, will not easily translate to use in humans because of the difficulty of testing the safety of a live-attenuated HIV vaccine.

The absence of a tractable small-animal model is one factor in our failure completely to understand AIDS. Among the many important unanswered questions are the following. How does HIV cross mucosal surfaces such as those lining the mouth, vagina and anus? What are the cells that support primary HIV replication and what molecular and/or immunological factors determine the outcome of viral infection at the primary site? How does HIV spread from its primary site in a human, and how is its spread regulated? Are some strains of the virus more virulent than others? And how does HIV kill cells?

Intimately related to these questions about HIV are related issues such as the nature of the mucosal immune response to HIV; the ability to generate protective mucosal immunity against HIV; the nature of the systemic immune response that limits acute HIV infection; whether HIV can be eliminated from the body after infection; and the precise role of T cells and their products in attacking HIV. Sadly, the list of unanswered scientific questions is much longer than this sample. Although partial answers exist for several of these questions, firm answers on which to base specific therapeutic strategies or vaccine approaches are needed to provide the foundation for the next steps.

Lessons from polio

It is worth comparing AIDS to polio, another major disease for which a huge funding programme was central to eventual control. Poliomyelitis was a devastating epidemic disease in the middle of the twentieth century. Much of the research on it was supported by the March of Dimes/National Foundation for Infantile Paralysis, which funded a broad range of science, not just work that appeared to be directly related to the problem. That strategy paid off when Enders, Weller and Robbins from Harvard University found that poliovirus could be propagated in human embryo cells in culture. (This work subsequently won the researchers the

Nobel prize.) At the time, their studies were not focused primarily on polio but rather on cell culture and viruses in general. But this work provided the stimulus for Salk to develop a 'killed' polio vaccine. The efficacy of this and other vaccines (including the live-attenuated Sabin vaccine) eventually led to the control of the disease.

What is the relevance of polio to the current AIDS epidemic? In my view, the conquest of polio provides important lessons. For example, until Enders and his colleagues succeeded in growing the poliovirus, using basic tools of cell culture and viral isolation, the disease could not be controlled. The virus was isolated in a laboratory studying other viruses and cells. Thus the new finding appeared in an unlikely place and illustrates how basic science often produces answers to unexpected but urgent questions.

Another point to emerge from this story is that, because poliovirus vaccines were effective, it became unnecessary for scientific policy-makers to emphasize continued basic research on polio. Consequently, there are still gaps in our scientific understanding of the mechanisms by which the poliovirus causes paralysis, partly because of a loss of interest in the problem after generation of the vaccine. For example, we do not know which primary cell supports poliovirus replication, how the virus spreads from these primary sites, or the nature of the mucosal immune response. Now that the nature of the poliovirus receptor is known and a transgenic mouse model of the disease is available, it has become more feasible to provide answers to these and other questions. But it was not critical in the 1950s to understand poliovirus pathogenesis to control the disease. The vaccines worked!

How does AIDS differ from polio? Polio causes an acute viral infection, while in AIDS there is no elimination of HIV following primary infection. Another big difference between HIV infection and polio is that, whereas injected poliovirus immune serum as well as inoculated killed and/or attenuated vaccines all prevent poliovirus replication in the infected host, it is increasingly apparent that, for HIV, generating an effective immune response by the multiple approaches used so far does not appear to prevent or control the replication of HIV. For this reason, in contrast to the situation that existed for poliovirus in the 1950s, we need to emphasize basic research related to AIDS. The critical breakthrough for poliovirus involved isolation of the virus: HIV has already been identified, but to outwit this persistent viral infection we need to learn much more.

To summarize, we approached the problem of AIDS using the paradigm of poliovirus and other acute lytic (cell-

destroying) viruses: isolate the virus, develop a vaccine and prevent the disease. The additional research strategy of the 1980s and 1990s has been the development of antiviral drugs. But although this general approach was reasonable for the first decade of AIDS research, we must now admit that these familiar paradigms have not shown great promise in solving the problem. We still have too many serious gaps in our fundamental knowledge to know how to prevent and treat AIDS, and must return to a broader base to study the scientific questions confronting us.

Where do we go next?

I wish I could answer this question. It is not difficult to point to gaps in knowledge. It is very difficult to predict, however, what leads will result in a 'breakthrough' cure or a preventive vaccine. Having acknowledged this reality, there are some general points to be made.

First, I believe that we need to broaden our definition of 'AIDS-related' research. Biomedical science is not a linear pursuit. Sometimes, the answers to a problem are discovered in the most unlikely and unpredictable places. AIDS is a novel disease requiring new paradigms and a new conceptual framework regarding persistent viruses and the host response. Our current predicament requires us to redouble our commitment to fundamental AIDS-related research.

How might this be done? One answer might be to make funding available for studies by investigators in other fields that are likely to provide general insights into pathogenesis and host response that could have a bearing on AIDS. Lymphocytic choriomeningitis virus in mice, to cite one example, provides an excellent model for studying viral persistence and the host response. We should also acknowledge that studies with different viruses, bacteria, fungi and other organisms might be relevant to a fundamental, unsolved problem in AIDS. We clearly need to continue to do most of our studies with HIV, but we must consider studies on other infectious agents and on microbial pathogenesis in general.

Second, we need to emphasize the basics of the science directly relevant to HIV and AIDS. For example, we should expand studies on AIDS pathogenesis and host immune response. For instance, we might entertain research proposals designed to approach pathogenesis through the study of early events of HIV infection, including studies of macrophages and dendritic cells, regulation of mucosal immunity, T cells and lymphokines in the search for new information about how the immune system reacts when confronted by HIV. These difficult, critical questions are essential for providing important information about how the host can assist in controlling or eliminating the virus.

Third, we should expand our studies of opportunistic infections such as cytomegalovirus, mycobacterium avium complex, tuberculosis and toxoplasmosis that claim the lives of so many AIDS patients, and, by increasing funding, encourage more investigators to enter these areas. Because many opportunistic organisms are more difficult to study than better-established model organisms, we need to provide longer-term support for high-quality proposals in this area.

Fourth, we should, of course, continue to look directly for a new drug or effective vaccine. However, in the haste to find the magic bullet or miracle vaccine, the real challenge is to put aside politics and the illusion of easy answers so that we can concentrate on studies that offer a real possibility of working, too many studies provide little scientific reason to believe that anything will be achieved. Perhaps the main point in the clinical evaluations is to have a carefully thought-out process that reviews data, has broad input and minimizes wasteful programmes that have little or no scientific merit. In our zeal to control AIDS, we have invested enormous resources in the search for drugs and vaccines. This may have been reasonable 10 years ago, but is no longer.

Final thought

When searching for the cure of a human disease, both basic research and medical engineering are critical. If one understands fully how a disease works, then it is more likely that a cure might be engineered or produced. But if fundamental scientific gaps exist, then broader based scientific research is needed.

Our capacity to move quickly to identify HIV as the cause of AIDS came from a commitment to viral oncology and retrovirus research made in the late 1960s and 1970s. Thus it was a commitment to attack cancer that led to the identification in the 1980s of the nature of the AIDS virus. A treatment or preventive strategy for the disease is as likely to come from fundamental discoveries in fields other than AIDS research as from those targeted for AIDS.

Paradoxically, by targeting too narrowly, we may slow down progress in combating AIDS. We must not compromise research in other areas of basic science at the expense of these directed programmes. This would risk eliminating the research project that holds the ultimate answers to a critical piece of the puzzle. We must give serendipity (and reasoned scientific redirection) a chance to join the war on AIDS. □

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Can evidence ever be inconclusive?

The belief that stark conclusions can be made less definite by suggesting that inconclusive evidence is relevant, but in some manner undefined, can lead to trouble.

EVERY so often, readers of the journals come across articles whose titles begin with the weasel words "Evidence of..." or "Evidence for..."; they should be appropriately cautious of the text that follows. In the interests of plain speaking, this journal does its best (not always successfully) to ban these forms of words, which have been devalued by ambiguous and often tendentious usage. Their precise meaning has most recently arisen in connection with Fermilab's announcement a few weeks ago (see *Nature* **368**, 805; 1994) of what has widely been described as the "discovery of the top quark": both the laboratory's announcement and, it is said, the article submitted for publication have titles that begin in just this way.

In the case of Fermilab, mercifully, there are ample (if unpublished) indications of what the words are meant to mean. For at least a week before the announcement, the laboratory had agonized about the significance of the data it had gathered. An announcement was supposed imminent at the spring meeting of the American Physical Society, but was then put off. In the end, the laboratory went out of its way to emphasize that the data available for analysis were less conclusive than it would have wished. It is not merely that the estimated error of the estimated mass of the top quark is uncomfortably large (roughly 10 per cent), but that it is conceivable that the measurements may be differently interpreted.

What seems to have decided Fermilab in favour of publication is that its data are more persuasive than others have so far produced, and that it is likely to be some time before anybody will be able to do substantially better. So in this case "Evidence for..." means something like "The best evidence yet...". Nobody who has followed the laboratory's statements on just this point can doubt that it has acted responsibly, seeking not to seem to make a false claim. So why did it not use the phrase "The best evidence yet..." or, if persuaded that only latinate constructions appeal to the journals with which it deals, "Further evidence for..."? One version of a press announcement uses the equally proper "New evidence for...".

None of this implies that "evidence for" should never be used, but merely that it must be sufficiently well qualified for its meaning to be clear. Thus "evidence from seismic observations for a molten core" makes sense, but "seismic evidence for a molten core" is better. The improper usage is that in which the words are used to refer to a conclusion that is only partly

supported by the self-same evidence.

Because people these days read so little, the rhetorical stratagem succeeds once the unsupported conclusion is embodied in a bibliographically retrievable title, and there are of course other ways of doing that, as in "Possible theories of cold fusion" (Fleischmann, M., Pons, S. & Preparata, G. *Il Nuovo Cimento* **107A**, 143; 1994), against which journals should also be on their guard.

Yet "Evidence for..." persists. Conversations (or, more accurately, arguments) with authors reveal a profound misunderstanding on this point. If an article with a fine declarative title should run into trouble with its referees on the grounds that the text does not fully support the conclusion, but the work itself is inherently interesting, there is every likelihood that the author will seek to keep the original title, prefacing it with "Evidence for...". The misunderstanding seems to be that the weasel words betoken evidence that falls short of proof. The most effective way of concluding these conversations is to offer the alternative, "Inconclusive evidence for...".

The same misunderstanding seems to underlie a dispute that has arisen over an important article published last year under the title, "Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion" (*Science* **262**, 1032–1034; 1993). This is an important article, due to J. B. Kerr and C. T. McElroy of Environment Canada, based at the Atmospheric Environment Service, Toronto, who have developed and operated for the past four years a ground-based instrument for the measurement of ultraviolet radiation in the 300-nm region (from 290 nm to 325 nm).

In the argument about the consequences for living things of ozone concentrations in the stratosphere, one obvious logical gap has been the absence of information about the intensity of ultraviolet-B radiation at the surface of the Earth. While the concentration of stratospheric ozone (which absorbs at 300 nm) must be one of the chief determinants of the ultraviolet intensity, absorption in the troposphere (by particulate pollution, for example) and even the weather can affect day-to-day intensity on the ground.

Kerr and McElroy have designed an instrument that is excellently suited to the accumulation of an understanding of ultraviolet intensity on the ground. First, it measures intensity at intervals of 0.5 nm throughout the spectral range, routinely scanning the defined range twice each hour. The same station at Toronto also operates a

system for the direct measurement of stratospheric ozone, so that the effect and its supposed cause can be directly correlated.

So what can have gone wrong? By "large upward trend", Kerr and McElroy meant what they say in their abstract and their text, that there is an upward trend of ultraviolet radiation to match the measured downward trend of stratospheric ozone in the past four years. But do four years constitute a sufficient interval of time to prove a trend, especially when ozone concentrations in one of the years concerned were exceptionally reduced by debris from the Pinatubo volcano, and when ozone data for the winter of 1991–92 are missing (because the ozone instrument was away for calibration)?

The issue has been seized upon by Dr S. Fred Singer, the long-standing thorn in the flesh of the environmentalists who is now director of the Science and Environmental Project in Washington, DC, but otherwise a professor at the University of Virginia. His complaint is not so much that four years (of which two are exceptional) are not enough on which to base claims of a trend, but that the appearance of a statistical trend is almost entirely accounted for by the exceptionally high ultraviolet measurements towards the end of the period.

The argument, apparently about to be rehearsed by a published criticism of Kerr and McElroy and a rebuttal, is in reality an irrelevance. What matters about their work is that there is now an instrument of proven reliability with which, it is hoped, ultraviolet intensities elsewhere than at Toronto will be tracked. Moreover, their data do indeed show a good correlation between ozone concentration and ultraviolet intensity at the surface, irrespective of season or time of day; analysis will in due course reveal how important are the confounding factors (particulate pollution and the like).

But Singer is surely right to protest that the trend now claimed cannot, on the evidence, be a proof that ultraviolet intensities are increasing in the global and secular fashion implied. (When the article appeared, there was a great deal of publicity for the estimate that winter-ultraviolet intensities appeared to be increasing at 0.4 per cent a year, even though they are negligible compared with summer intensities at 300 nm). But the authors say that their rebuttal will in part consist of saying that they did not claim a "large upward trend", but only "evidence for" one. The disputed words have all the appearance of having been added to satisfy a referee, but so what?

John Maddox

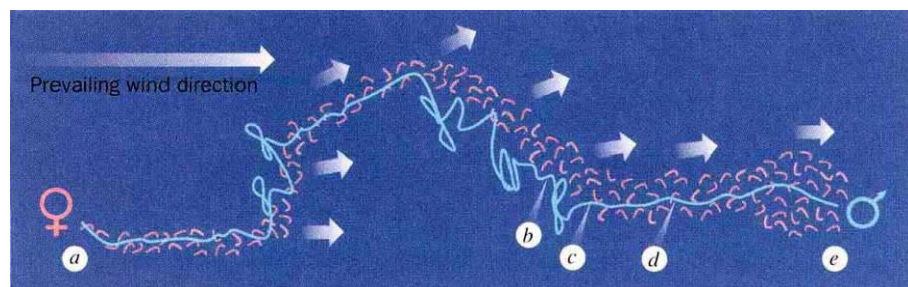
Moth flights of fancy

Tristram D. Wyatt

To mate with a female, male moths must find her by flying upwind, tracing a stream of highly dilute pheromone over potentially great distances and despite shifting winds. As described at a meeting in March* and in two papers — one on page 142 of this issue¹, the other forthcoming² — new work provides insight into this feat: in consequence we can now relate upwind flight of moths to the fine structure of pheromone plumes. The meeting,

so the similarity of their results gives some confidence that the hypothesis is sound. Moreover, the findings are likely to apply both to other insects and to female moths (which show very similar upwind flight behaviour to plant host odour^{6,7}, and respond to sex pheromone in the same way as males if given 'male antennae' by imaginal disk transplant⁸).

Working with male *Cadra cautella*, Mafra-Neto and Cardé found that slowly



Idealized birds-eye view of a flying moth, showing how surge-cast-surge behaviour might lead the insect to the pheromone source (after ref. 4). Instantaneous wind direction is indicated by the arrows; pheromone filaments are in pink. *a* is the pheromone source; *e* where the male starts. At *d*, the moth is flying straight upwind into the plume, responding to frequent contact with pheromone with reiterated surges. At *c*, it emerges into clean air and responds with casting flight. At *b*, regaining contact with pheromone yields a brief surge.

though, was wide-ranging, covering insect pheromones all the way from molecules to evolution (of which more below).

The first identification of an insect pheromone some 40 years ago revealed the agent that attracts male moths to females. But that left open the orientation mechanism, which must explain why moths fly upwind best to intermittent or turbulent plumes (characteristic of real plumes³), and show side-to-side casting behaviour in both clean air (after contact with pheromone) and in uniform clouds of pheromone⁴. The current consensus is that upwind orientation combines two programmes, optomotor anemotaxis and self-steered counterturning^{4,5}. Both behaviours are initiated by contact with pheromone, casting flight being produced on its loss. Baker⁴ hypothesized that the two behaviours could be explained by tonic (casting) responses overridden by intermittent phasic (upwind bursts) responses to antennal contacts with filaments of the pheromone plume.

Mafra-Neto and Cardé¹, and Vickers and Baker², have now come up with experimental evidence that strongly supports this idea. Both groups show that the moth can respond to individual filaments (odour-laden strands) of pheromone in the plume. The two studies used different species of moth and different techniques,

pulsed plumes ($0.6 \text{ pulses s}^{-1}$) or a continuous ribbon plume led to slow upwind progress with much zigzagging. Males flew faster and with a straighter path in quickly pulsed (4.7 s^{-1}) or turbulent plumes. Males in casting flight responded to contact with a single 0.25-s pheromone puff by surging ahead. The near-identical response of casting male *Heliothis virescens* to single pheromone puffs was used by Vickers and Baker to produce a template of cast-surge-cast behaviour. In this species the slightly tortuous tracks in response to a plume of 4 pulses s^{-1} can be explained by a series of casts (clean air) and surges (pheromone pulse) matching their template. Moths flew nearly straight upwind tracks in response to 10 pulses s^{-1} .

Vickers and Baker take the experiments one step further by using an antenna (from a second male) attached by Velcro across the head of the moth to give an in-flight electroantennogram (EAG). The 'cyclops' antenna responds with an EAG depolarization, read via fine wires, when pheromone puffs hit the flying moth, and these can be matched to surges in forward flight. When filament contacts cease, giving 'silence' in the EAG for more than about 0.3 s , casting starts.

Different response times to pheromone contact and loss may explain^{4,6} why males of some species (*H. virescens* and *C. cautella*, for instance) fly relatively straight paths upwind, compared with the more meandering tracks shown by others

(for example *Grapholitha molesta*⁹). With a given pheromone pulse rate, species with longer latencies to casting behaviour on loss of pheromone will give straighter upwind flight, as there is more chance the male will hit the next pheromone filament before casting is initiated. These reaction latencies correspond well to the inter-turn durations of these species^{4,6}.

What is the function of the surge-cast behaviour? Baker suggests that to track the shifting plume the moth needs to have a fast surge response to filament hits but, equally, a fast decay so that the moth starts casting on flying into the following void of clean air (see figure). Casting will increase the chance of hitting the next filament.

The structure of real plumes was discussed at the meeting. Advances in technology allow real-time, single-cell recording of moth antennal receptors in the field (J. N. C. van de Pers, VDP Laboratories; A. K. Minks, IPO-DLO, Wageningen); these records show fluctuations in spiking, corresponding to contact with pheromone filaments interspersed with contact with clean air, at the fine timescale previously predicted by ionized-air tracers and other techniques³.

Neurophysiological studies paralleling behavioural analysis are beginning to hint at neural analogues of the 'phasic' and 'tonic' behavioural systems. Pheromone-sensitive interneurons have been discovered, within the antennal lobes of moth brains, which are able to follow phasic stimuli applied to the antennae with high fidelity up to frequencies of about 10 Hz (ref. 10). Elsewhere in the brain, pheromone-responsive neurons, whose processes descend in the ventral nerve chord towards thoracic ganglia, have been identified which show long-lasting excitatory (tonic) activity and state-dependent¹¹ or flip-flopping^{12,13} responses to repetitive stimulation, reminiscent of certain aspects of the behaviour. The role that such neurons might play in olfactory-mediated locomotion is being hotly pursued in several laboratories.

Great progress has been made at the level of pheromone reception by antennal chemosensory neurons, a topic also covered at the meeting: we now have clear evidence that the chemoelectrical transduction process is mediated by G-protein-coupled reaction cascades. Ironically, though, the pheromone receptors themselves remain elusive (H. Breer, University of Stuttgart-Hohenheim). Stopped-flow experiments have shown that the primary reaction in antennal cells elicited by very low pheromone doses is a rapid and transient increase in inositol trisphosphate (IP_3) concentration; the pheromone-induced 'pulse' of IP_3 is supposed to trigger the electrical response. The rapid termination of the transduction cascade is of particular importance for

* 1st International Symposium on Insect Pheromones, Wageningen, The Netherlands, 6–11 March 1994.

sensory cells that respond to repeated stimulations, for example in a pheromone plume. The 'turn off' reaction is mediated by phosphorylation of specific proteins, probably activated pheromone receptors; it may involve an arrestin-like protein, now cloned from antennal complementary DNA libraries of two insect species.

A variety of antennal binding proteins has been identified by molecular cloning, and it seems that they may each have a specific function in the perireceptor events of olfaction. Characterization of the binding sites of cloned pheromone-binding proteins (PBPs) by photoaffinity radioligand labelling explains the specificity of PBPs for a certain pheromone, and emphasizes the importance of double bonds in the pheromone molecule previously observed at the behavioural level (G. D. Prestwich, SUNY). Quite how PBPs act in the system is still not clear, but it looks likely that the pheromone is presented to the receptor by PBPs.

Evolutionary biologists are offered unparalleled opportunities by insect pheromone systems, in which communication behaviour is combined with large data sets and an ability to study individual genes and gene products (including the PBPs in males and the enzymes of pheromone biosynthesis pathways). How major shifts in pheromone blends can occur was shown by a serendipitous observation of change in a laboratory population. The change, due to a single recessive autosomal gene, resulted in *Trichoplusia ni* females producing a radically altered blend; selection of conspecific males responding to the new blend was rapid (K. Haynes, University of Kentucky). This result, and the increasingly successful use of pheromones for mating disruption to control moth pests, raises questions of the possible development of resistance given the enormous selection pressures now being generated in the field. □

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Sex and deleterious mutation

Alexey S. Kondrashov

SEX, which involves the alternation of meiosis and gamete fusion, is a rather inefficient means of self-propagation as compared to asexual reproduction, where offspring stem only from a mitotically produced cell. Yet sex is ubiquitous among eukaryotes. Why?

One plausible reason for the origin and maintenance of sex is its ability to reduce the mutation load if deleterious mutations interact synergistically — that is, if consecutive mutations lead to an increasing decline in relative fitness (epistatic selection). Previous analyses of this idea have assumed that the genomic deleterious mutation rate μ is the same in both sexes, but it has emerged that male gametes may generally be more subject to mutation than female gametes. Does it still make sense for females to practice sex even then? Rosemary Redfield addresses this intriguing question on page 145 of this issue¹.

Some of the conclusions from her computer modelling study are unremarkable, others striking. If addition of a mutation always leads to the same decline of relative fitness — the fitness of an individual with k mutations is $(1-s)^k$, where s is the coefficient of selection against a mutation — sex does not reduce the mutation load even when μ is the same in both sexes. So if male gametes carry more mutations, asexual females gain an advantage by not contaminating their progeny's genomes.

However, sex can still be advantageous with epistatic selection, although under more restrictive conditions. With the extreme mode of synergistic epistasis, truncation selection — all individuals with no more than T mutations have the highest fitness, while all the rest die (see Fig. 1 on page 145) — sex can have a more than twofold advantage even when the ratio of male to female deleterious mutation rates, α , is much higher than one. This requires μ to be about 2 or more (although, surprisingly, its further increase does not make a difference) and high T , which implies that individual mutant alleles are only slightly deleterious. Redfield does not report any data for T of more than 30, but extrapolating her results from Fig. 4 (on page 146) one can conclude that with μ greater than 2 and T of 100, sex wins even when α is 10 (which may be its highest realistic value).

This result has a simple explanation. Epistatic selection reduces the population variance of the number of mutations per genome, and sex is advantageous because it restores this variance. This effect grows with T , because sex simply tries to make distributions of different mutant alleles independent, and thus cannot make the

variance of their genomic number more than about the value of T (when rare mutant alleles occur independently, their genomic number has Poisson distribution with the mean close to T). The same is true under other, less extreme, forms of synergism, although strict truncation leads to the largest advantage.

High values of α have so far been reported only for mammals — perhaps the only group where the maintenance of sex is not an issue, because both a female and a male gamete are necessary for reproduction, due to genomic imprinting². In *Drosophila*, α is perhaps close to 2 (ref. 3). However, at least moderate increase of the mutation rate in males may be common because males produce smaller but more numerous gametes and thus the male germline passes through more cell divisions than the female.

By my count, 20 hypotheses trying to explain the evolution of sex have been proposed to date⁴. The mutational deterministic hypothesis which invokes epistatic selection against deleterious mutations is not the only plausible one. It is attractive, however, because mutations are ubiquitous, and thus may be the sole factor underlying the evolution of sex, and because it is relatively easy to test. It was already clear that the mutational deterministic hypothesis can be correct only if μ is greater than or equal to 1 and there is substantial synergistic epistasis. But Redfield's results now show that μ should be measured in both sexes and, if in some species α is much greater than 1, that the hypothesis requires μ to be more than 2 and mutations to be only slightly deleterious.

As well as its importance in the evolution of sex and related phenomena (recombination, breeding systems and so on), μ may be central to the evolution of mate choice, quantitative variability and ageing⁵. Because most deleterious mutations have only slight effects, and can hardly be efficiently removed in civilized human societies, μ is also of obvious importance to medical genetics. So μ , estimated separately for males and females, emerges as one of the key parameters in population genetics. Measuring it is difficult, because slightly deleterious mutations cannot be detected individu-

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ally, but it is not impossible, as Mukai was the first to show 30 years ago⁶. Such experiments have yielded μ of about 1 or higher⁷, but more data are necessary.

The rates of molecular evolution of neutral sequences, as well as direct data⁸, show that in mammals the mutation rate per nucleotide per generation is about 10^{-8} , which implies some 100 mutations

per diploid genome. Of course, this gives only an upper estimate of μ , because only a fraction of all mutations are deleterious. At the moment, we cannot rule out any value of that fraction between under 1 per cent and over 50 per cent.

It is time to realize that the controversy about the factors involved in the evolution of sex and several other phenomena will

not be resolved by theoretical arguments. It is clear what data are necessary, and experimentalists should not pass a unique opportunity to make 19 hypotheses obsolete at a single stroke. □

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SPELEOLOGY

Creatures from the black lagoon

If laboratory work sometimes seems claustrophobic, pity the diver below, almost submerged in the sulphurous waters of Movile cave in southeastern Romania. The cave has no natural entrance and lies under a thick cover of wind-blown silt; it was discovered only in 1986, when it was broken into by shaft construction, and its former isolation is indicated by the lack of the radioactivity from the Chernobyl nuclear disaster that is usually present in Romanian caves. This way, researchers gained access to a unique ecosystem which has been isolated from the surface for millions of years.

Despite the absence of sunlight, the cave supports a rich invertebrate community. Forty-seven species of cave-adapted invertebrates have been discovered there, 31 of which are unknown elsewhere. The basis for the ecosystem appears to be sulphur-metabolizing bacteria, which synthesize organic matter using energy obtained from the oxidation of hydrogen sulphide — the cave contains white tufts of a fungal bacterial mat, especially in rooms enterable only through a subterranean lake. The food chain starts with H_2S and CO_2 , with the resulting acids buffered by the limestone bedrock. These chemoautotrophic bacteria in turn feed other bacteria and fungi that support the cave animals.

Depending on whether they live in anaerobic (water-filled) or aerobic (air-filled) limestone cavities, 'sulphur bacteria' can metabolize sulphur in one of three different ways: oxidize H_2S to sulphuric acid, as in Movile; reduce sulphates to H_2S using energy from external organic matter; or oxidize H_2S to native sulphur.

The study of these bugs is of more than just academic interest, as was amply proved by the conference* at which the descriptions of life in Movile cave were a highlight (S. M. Sarbu and T. Kane, Univ. of Cincinnati). Sulphide-oxidizing bacter-

ia such as *Thiobrix* in well water from limestone aquifers can clog irrigation pipes (R. Brigmon, Univ. of Florida; H. Martin, Univ. of Georgia). And their sulphate-reducing counterparts can foul platinum electrodes that are used to evaluate the oxidation potential of



C. Lascau/S. M. Sarbu

swampy green colour and contained catfish and mosquito fish, the bottom layer was clear and supported shrimp and blind cave fish. Sunlight was absent at the bottom and in the brackish strata. These intermediate layers, generally brownish in divers' lights and in samples, have remarkable concentrations of H_2S , which increase with depth to more than 25 mg per litre, the limit of the test equipment.

The luckless divers in this well found that their chromium-plated equipment turned black, they felt nauseated, and their exposed skin felt burned. They were understandably surprised to discover that the water contained living larvae of the phantom midge, evidently a creature made of sterner stuff. The cause of the hostile conditions seems to be a filamentous sulphate-reducing bacterium, probably a species of *Desulfovibrio*, which drapes the rocks and underground tree limbs there in a thick white coating.

The dissolution of limestone to produce most caves results from carbonic acid derived from soil gases. But some of the world's largest caves are believed to have been formed by sulphuric acid resulting from the action of sulphur bacteria. At Carlsbad Caverns National Park, it seems that H_2S from the oil fields of West Texas migrated northwards and upwards to form sulphuric acid that etched out the caves (C. Hill, Hi Tech, Albuquerque).

In the same park, recent exploration of the great Lechiguilla cave has expanded its known size and revealed decorations of exquisite beauty (K. Cunningham, US Geological Survey), many of which owe their origin to formerly nauseating processes mediated by sulphur bacteria. Deposits of native sulphur, so massive they are measured in tonnes, remain in the cave today as clear evidence of the former activity of the bacteria.

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ground water. Their presence in limestone aquifers can be diagnosed from characteristic concentrations of hydrogen, an intermediate in the redox reaction, and detectable by gas chromatography (D. Lovley, US Geological Survey).

Natural limestone wells in coastal areas sometimes also show evidence of sulphur activity, resulting there from the interaction of sulphate-bearing sea water with infalls of forest litter. For instance, the northern Bahamas show a zone where organic material becomes neutrally buoyant at the interface between fresh water above and denser salt water below (F. Whitaker, Univ. of Bristol). This promotes the growth of sulphate-reducing bacteria and causes a solution nip to cut into the limestone.

An even more dramatic case occurs at a natural well, Conote Verde, 4 km inland from the coast of Yucatan (W. Wilson, Karst Environmental Services, Florida). When the well was visited by divers in the autumn, the water was stratified: fresh in the top 8 m, salt below a depth of 30 m, and with various brackish concentrations in between. The top layer was a

* Breakthroughs in Karst Geomicrobiology and Redox Geochemistry, University of Colorado at Colorado Springs, 16–19 February 1994.

Genes in search of functions

André Goffeau

FOR millennia, the yeast *Saccharomyces cerevisiae* has been of great service to mankind as an agent in brewing and bread-making. In the late twentieth century it is offering more still, in the form of a model eukaryote for geneticists. The sequencing of its genome proceeds apace. But that enterprise has a yawning hole in the middle in that many of the genes turned up by sequencing have no known function.

Now, however, we may be on the point of finding out what thousands of yeast genes do. One of the steps forward that has made that prospect possible is described by Michael Snyder and colleagues (N. Burns *et al.*) in the May issue of *Genes and Development*¹. Their approach consists of systematically knocking out yeast genes by random introduction of a β -galactosidase (*lacZ*) marker gene. The phenotypes of the resulting single-gene disruptants provide clues as to the physiological functions of the gene products and their intracellular location.

The approach can be applied on a large scale: hundreds or even thousands of genes can be handled in parallel. Furthermore, the sequences of the genes concerned need not be known beforehand (before disruption). The report is especially timely, given that the entire sequence of the yeast genome is within shooting distance. The nucleotide sequences of half of the yeast genes should be known within a year, and the total molecular structure of the yeast genome will hold no secrets by the end of 1996 (see table).

The next frontier is now clear: for a large proportion (well over half) of yeast genes we will have a DNA sequence but no other experimental data. Several projects are under way to identify their biochemical and physiological functions, notably a pilot study by P. Slonimski, M. Bolotin and others supported by the European Biotechnology Programme (which is one of the main contributors to the genome sequencing project). The pioneering approach of Burns *et al.* has come out of the blue, as it were, and outside the framework of any official genome analysis programme.

The method stems from the association of two very efficient recombination processes: random insertion of the Tn3 transposable sequence in *Escherichia coli* and the homologous recombination in yeast. Briefly, the procedure has the following steps. (1) Random *lacZ* insertions by Tn3 in a yeast DNA library amplified in *E. coli*. (2) Isolation and linearization by *NorI* of the fused yeast DNA *lacZ* fragments. (3) Transformation of diploid

yeast cells and substitution, by homologous recombination, of a *lacZ*-labelled fusion product for a parental yeast gene. (4) Identification of yeast genes expressed during vegetative growth and sporulation by means of a colour assay using X-gal as substrate. (5) Intracellular localization of the fusion proteins by indirect fluorescence using anti- β -galactosidase antibodies. (6) Plasmid rescue and sequencing of the 5' end of the gene interrupted in-frame by *lacZ*.

None of these techniques is totally novel and there is another report in the press² of the successful use of a randomly fused yeast DNA library. The method of Burns *et al.*, however, is a particularly cunning combination of several approaches and their report is the first to demonstrate the feasibility of large-scale and systematic analysis of a variety of functional traits of all 6,000–7,000 yeast genes.

Only a few phenotypic tests were used to characterize haploid growth and meiotic development. Nonetheless, as applied to 2,800 yeast strains expressing fused genes, the technique has already yielded much information. For instance, out of the estimated 93–135 genes specifically induced during meiosis and sporulation, 39 *lacZ* fusion genes have been thoroughly analysed. Among these, 20 were previously unknown genes, most of which turned out to be inessential. Quite surprisingly, the expression of Y' telomeric elements as well as of ribosomal RNA genes was induced during sporu-

lation. Other new proteins were found to affect sporulation, such as a yeast homologue of the bacterial MutS protein involved in mismatched base repair and a homologue of a mammalian copper-transporting P-type ATPase responsible for Menkes disease.

Other findings concern the location of some of the fusion proteins: 245 of them localize to discrete subcellular locations such as the nucleus, mitochondria, endoplasmic reticulum, cytoplasmic dots (probably Golgi and Ty1-containing particles), spindle pole bodies, or microtubules. Several of these features may be of general interest, because many of the fusion proteins observed in subcellular bodies correspond with previously unidentified genes. Having been tagged by their 5' DNA sequences, they will be recognized once the full nucleotide sequence of the gene is available or should a homologous sequence be found in man or another eukaryote.

The method does have some drawbacks. The discriminatory power of indirect fluorescence is limited by the resolution of light microscopy, although this problem might be overcome by the development of an immunodetection procedure for the electron microscope. Another drawback may prove more serious: the method implies fragmentation of the gene product (the encoded protein) and possibly the promoter region (which might reduce or modify the expression of the protein). Both of these factors might affect the subcellular location. The authors address this issue, giving several examples of correctly located soluble proteins. Yet at least some proteins — especially membrane proteins — are bound to be mislocated, which makes it

Sequencing the yeast genome

Chromosome	Length (kb)	Coordinator	Affiliation	Release date*
III	314	S. Oliver	UMIST, Manchester	Mar. 92
XI	666	B. Dujon	Pasteur, Paris	May 94
VIII	550	M. Johnston	Washington Univ., St Louis	July 94
I	235	H. Bussey	McGill Univ., Montreal	Sept. 94
V	550	R. Davis	Stanford Univ.	Dec. 94
IX	440	B. Barrell	Sanger C., Cambridge	94
II	820	H. Feldmann	Munich Univ.	94
VI	270	Y. Murakami	Riken, Tsukuba	Early 95
X	720	F. Galibert	CNRS, Rennes	Early 95
XII (right arm)	750	M. Johnston	Washington Univ., St Louis	Early 95
IV (right arm)	500	R. Davis	Stanford Univ.	Mid 95
XIV	810	P. Philippsen	Basel Univ.	Late 95
XIII	910	B. Barrell	Sanger C., Cambridge	95
IV (middle)	540	B. Barrell	Sanger C., Cambridge	95
VII	1,150	L. Tettelin	Louvain-la-Neuve Univ.	96
XV	1,150	B. Dujon	Pasteur, Paris	96
XII (left arm)	450	J. Hoheisel	DKFZ, Heidelberg	96
XVI (left arm)	592	H. Bussey	McGill Univ., Montreal	96
IV (left arm)	600	C. Jacq	ENS, Paris	96
XVI (right arm)	450	B. Barrell	Sanger C., Cambridge	96

* Expected release date of the full sequence; that of chromosome III has already been published³ and that of chromosome XI has been released⁴. Further subdivision of the longest chromosomes between different groups is being considered to speed up completion of the sequence.

essential to confirm location by an independent method.

Following the tradition of openness with information and materials maintained by the yeast scientific community, it seems that the fused gene library will be made freely available. Indeed, the authors say that their "results will allow researchers to determine immediately whether that gene is expressed at a specific time during the life cycle and whether its gene product localizes to a specific subcellular location". But a complete yeast genome library will require further analyses of about 30,000 transformants, of which only 2,800 have yet been screened.

Jacques Monod has said that what is true for bacteria is true for an elephant. I feel tempted to say that what is true for yeast is true for man. The unravelling of the human genome is a gigantic task, often said to require big, centralized sequencing and data-processing facilities. Yeast research, in contrast, is a comparatively

small science, carried out by some 4,000 scientists in many small laboratories around the world. The yeast genome is compact and efficient. Each chromosome contains fewer than 2 million base pairs but each 2,000-base-pair stretch encodes a protein of over 100 amino acids in length. Identifying the function of all these genes, many of which will have human homologues, is also a daunting task. Yet in the paper by Burns *et al.* we see how small science, imaginatively applied, can produce a solution that may render more impressive means obsolete. □

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GRAVITY

Holes in general relativity

Arlen Anderson, Jonathan Halliwell and Noah Linden

ONE of the outstanding challenges for theoretical physics is to construct a theory of the gravitational field that is consistent with quantum theory. Despite considerable efforts over the past decade or so, the problem remains unsolved. At a meeting in March*, theoretical physicists took stock of the present status of classical and quantum gravity.

The currently accepted classical theory of gravity, Einstein's general relativity, remains in extremely good shape. It is one of the most accurately tested of all physical theories — indeed, its prediction that bodies of different composition fall with equal acceleration has been confirmed to one part in 10^{12} (T. Damour, IHES, Bures-sur-Yvette), and there are proposals for a satellite test that will have a sensitivity of a part in 10^{17} . Last year's Nobel Prize for physics was awarded to Hulse and Taylor for the discovery of a binary pulsar whose precise timing allows tests of general relativity in conditions very different from any accessible within the Solar System. In principle, the data from the binary pulsar could provide 12 independent tests of the theory, but these have not yet been carried out. At present no experimental results indicate any deviation from Einstein's theory.

Despite this tremendous success for weak fields, a possible objection to the ultimate validity of classical general relativity is its prediction of singularities in

spacetime — points at which the theory itself breaks down and new physics is expected to come into play. In the work of Hawking and Penrose in the 1960s, singularities were identified by the incompleteness of geodesics: that is, as points beyond which the trajectories of freely falling observers cannot be extended. These points were believed to be associated with infinite curvature of spacetime, such as arises at the centre of black holes.

One answer to this was to assert that it didn't matter — all singularities in general relativity are hidden behind event horizons, and therefore irrelevant to physics outside the horizons. This is the 'cosmic censorship' hypothesis, and efforts to prove some form of cosmic censorship have led to basic changes in the conception of general relativity, switching from the language of differential geometry to differential equations. This follows the recognition that Einstein's equations can be solved even when geometric concepts such as geodesics break down (C. J. S. Clarke, Univ. of Southampton).

Related numerical work has revealed an unexpectedly rich structure in the formation of black holes, reminiscent of critical behaviour at a phase transition (J. Stewart, Univ. of Cambridge). It seems that the mass of a black hole formed from the spherically symmetric collapse of a massless scalar field is described by a power law with a universal exponent, provided a parameter characterizing the initial data exceeds a certain critical

value¹. Furthermore, at the critical value the scalar field exhibits a self-similarity. This work (confirmed for axisymmetric collapse of gravitational waves² and for a perfect fluid³) may throw a spanner in the works for cosmic censorship because it indicates that black holes of arbitrarily small mass can be formed and therefore that arbitrarily large curvatures will be visible outside the horizon.

Can quantum effects improve the situation by doing away with the singularities in the classical theory? It seems not. Years ago, Hawking showed that quantum field theory in a classical curved spacetime predicts the evaporation of black holes and subsequently reached the disturbing conclusion that information is irretrievably lost in the process. Now it seems that information may also be lost in spacetimes that contain closed timelike curves but no curvature singularities (S. Hawking, Univ. of Cambridge). This supports the argument that information is lost in black-hole evaporation and cannot be restored by a fully quantum theory of gravity, which presumably would only be important in regions of high curvature⁴.

The notion of information is often discussed in the context of black holes, but the full weight of Shannon's information theory has yet to be properly applied. Information theory has often been used in quantum theory in the past, and may lead to a more concrete understanding of the mechanism of information loss in black holes (J. H.).

Intertwined with the problem of quantum gravity is the question of interpreting quantum mechanics as applied to the entire Universe (quantum cosmology). Quantum mechanics, in the conventional Copenhagen approach, is interpreted by assuming the world to be separated into microscopic quantum systems (atoms, fundamental particles and the like) and macroscopic classical ones which may measure the microscopic systems. Such an assumption is clearly inadequate in a quantum theory of the entire Universe. The 'decoherent histories' approach to quantum mechanics, developed independently by Gell-Mann and Hartle, by Griffiths, and by Omnès, over the past ten years, is a formulation that does not rest on such assumptions, and may therefore be applied to genuinely closed systems such as the entire Universe (J. B. Hartle, Univ. of California at Santa Barbara).

In this approach, one considers sets of possible histories for the closed system, for example sequences of positions of a particle at successive times. When the quantum interference between such histories is negligible they may be assigned probabilities. One may then ask about the correlation between properties of the system at different times — for example, whether successive particle positions are

* *Classical and Quantum Gravity: A Survey Conference*, Isaac Newton Institute for Mathematical Sciences, Cambridge University, Cambridge, UK, 28–31 March 1994.

approximately correlated according to classical laws of motion. In this approach, therefore, the classical world of our experience and the Copenhagen approach to the quantum mechanics of microscopic systems are no longer assumed, but become calculable emergent features of an underlying quantum theory of the entire Universe.

The decoherent histories approach may also avoid one of the most serious difficulties with quantum gravity, namely the 'problem of time'. General relativity denies the existence of a preferred time variable, whereas quantum mechanics demands one in order to write down a Schrödinger equation. But in an approach to quantum theory based on histories, rather than on events at fixed moments of time, the need for a distinguished time variable is no longer so acute, because one

can extend the notion of a history to sequences of events whose time-ordering is not specified. Progress has been made in generalizing the decoherent histories approach along these lines, using ideas for quantum logic (C. J. Isham, Imperial College London), and it seems that a promising vein of investigation has been struck. □

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IMMUNOLOGY

Making class II presentable

Sandra L. Schmid and Michael R. Jackson

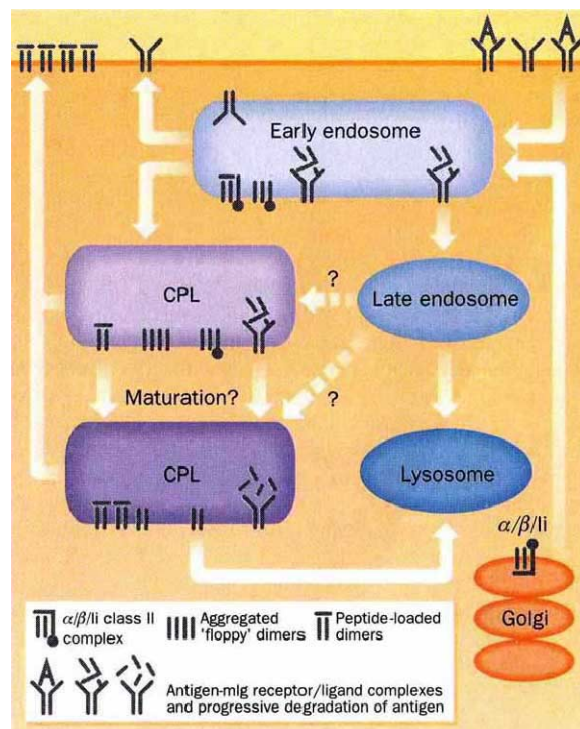
THREE papers in this issue^{1–3}, together with one in *Journal of Cell Biology*⁴ and unpublished observations by F. Castellino and R. Germain, combine to identify a unique endosome-related subcellular compartment in antigen-presenting cells (APCs). The importance of this compartment is that it is where class II molecules of the major histocompatibility complex (MHC) are loaded with their antigenic peptides.

Some principles underlying the new work are well established. Presentation of foreign antigens to helper T cells requires coordination of both the biosynthetic and endocytic pathways within specialized antigen-presenting cells. The endocytic pathway generates and delivers antigenic peptides to MHC class II molecules⁵ provided by the biosynthetic pathway. Class II molecules are α/β dimers which assemble in the endoplasmic reticulum with a third protein, invariant chain (Ii). The Ii blocks binding of endogenous antigens to the class II molecules and stabilizes the unoccupied α/β dimers while escorting them through the biosynthetic pathway to the endosomal compartment⁶. After removal of Ii in the acidic and proteolytic environment of the endosomal compartment, the class II molecules can bind peptides. Peptide-loaded class II complexes are then transported to the cell surface for inspection by T cells (see figure).

But how are these processing events orchestrated and where do they occur? One attractive idea has been that class II α/β /Ii complexes meet unprocessed antigen in an endosomal compartment and together move along the endocytic path-

way encountering progressively lower pH and more aggressive proteases. In this way proteolysis of Ii and the antigen coordi-

FIG. 1 Antigen processing and presentation. The new results described here show that the compartment(s) for peptide loading (CPL) of class II molecules in antigen-presenting cells (APCs) are biochemically distinct from conventional endosomal compartments. We propose that intracellular antigen presentation takes the following route. Newly synthesized class II α/β /Ii complexes are targeted from the Golgi to an early endosomal 'sorting' compartment (pale blue), identified as light density, Rab5- and Tfn-receptor- (TfnR) positive organelles. Degradation of the Ii begins in the acidic and mildly proteolytic environment of early endosomes and continues as the class II complexes are diverted to the CPL (purple) from the conventional endosomal pathway (darker blue) which is followed by bulk fluid phase markers. On arrival in a CPL of intermediate density, most of the Ii is degraded and class II molecules are mainly in their 'floppy' conformation, shown here as aggregated α/β chains receptive for antigenic peptide. The CPL 'mature' into a high-density compartment in which 'compact' peptide-loaded class II molecules accumulate. CPL are devoid of or contain only low levels of the early and late endosomal markers, including internalized HRP, Rab5, Tfn-receptors and mannose-6-phosphate receptors, and of the lysosomal markers, LAMPs. Receptor-bound antigens are also targeted to CPL from early endosomes and possibly from late endosomes (dashed arrows) during which time the antigens undergo progressive proteolytic degradation to generate a diverse range of antigenic peptides. When peptides are loaded onto class II molecules, the aggregated complexes dissociate and the peptide/class II complex is transported to the cell surface for presentation to T cells.



nately produces both unoccupied, receptive class II complexes and the antigenic peptide. As no specialized antigen processing or loading compartments were postulated, this model was consistent with the observation that, when transfected with class II α/β /Ii, most cell types can present antigen. Furthermore, employment of the full endocytic pathway in processing and loading explained how class II might acquire antigen from proteins irrespective of how stubbornly they resisted proteolysis.

This view now comes under serious challenge. The five groups concerned have used biochemical assays (the formation of SDS-stable class II/peptide complexes) or functional assays (the formation of class II/peptide complexes able to activate T cells) to identify a unique subcellular compartment in which class II peptide loading occurs (referred to here as the CPL, compartment(s) for peptide loading).

Two of the groups took advantage of the negative charge of endosomal compartments⁷ in their subcellular fractionation schemes by employing free-flow electrophoresis¹ or density gradient electrophoresis² to resolve the class II-containing intracellular compartments from typical early and late endosomes.

Pulse-chase experiments coupled to subcellular fractionation established that in these compartments biosynthetically labelled class II transiently accumulated before delivery to the cell surface; that Ii chains were absent or rapidly degraded; and that class II molecules first acquired peptides. Although CPL were biochemically distinct from early and late endosomes and lysosomes, they were accessible to endocytic tracers, which is consistent with their being a specialized subset of the endosomal compartment.

The CPL could also be resolved from conventional early and late endosomal compartments by Percoll density gradient centrifugation (refs 3, 4; F. Castellino and R. Germain, personal communication). B lymphocytes were fed unprocessed antigen for various times before fractionation^{3,4} and the CPL were identified in a high-density fraction in which functional, SDS-stable class II/peptide complexes could first (15–30 minutes) be detected. After 30–60 minutes the complexes appeared on the cell surface, and the peptide was able to stimulate T cells.

Morphological studies of the CPL in B cells^{1,3} revealed spherical structures with abundant internal membrane vesicles or membrane infoldings, much like multivesicular bodies, an intermediate in the conventional endosomal pathway. This morphology is similar to that of a class II-enriched compartment in human lymphoblasts⁸, which was found to contain a distinct subset of endosome/lysosome markers.

Together, these papers define distinct compartment(s) for peptide loading in antigen-presenting B lymphocytes. The CPL are distinguished from conventional early and late endosomes, including multivesicular bodies, by their subcellular fractionation behaviour, by their low or absent levels of certain endosomal markers, and by their relatively low accessibility to fluid phase markers. They are distinguished from lysosomes by the low level or absence of lysosomal membrane proteins.

Many questions remain to be resolved. First, are endosome-related CPL unique to APCs? Many cell types have specialized endosomal subcompartments; for example, adipocytes use such structures for storage and regulated surface expression of GLUT4 and other transporters, and neurons use them for retrieval and regeneration of synaptic vesicles⁹. Because there is no evidence for regulated transport to the cell surface from CPL, we think that CPL are more closely related to the series of transcytotic endosomes used by polarized cells for moving cargo between distinct domains of the cell surface¹⁰. As shown in the figure, class II α/β /Ii are probably delivered to early endosomes along a route followed by many immature

lysosomal hydrolases¹¹ and then rapidly sorted into the CPL. Cargo destined for conventional late endosomes is sorted into a distinct set of multivesicular bodies, which also apparently dissociate from the early endosome^{12,13}. We propose that the CPL undergoes a 'maturation' process during which Ii is degraded, class II assumes a 'floppy' conformational intermediate⁴ and, finally, peptide loading occurs.

Second, why is a specialized endosomal compartment needed for peptide loading? This question is especially relevant since virtually any cell transfected with class II α/β and Ii can present many different antigens to T cells. Although this presumably occurs by way of the conventional endosomal pathway, it is not very efficient. Perhaps CPL provide a particularly efficient means of presenting antigen.

One weakness of a process that relies on a proteolytic gradient to coordinate the availability of peptide-free class II and antigenic peptides is that Ii is presumably degraded in a narrow time window compared to the wide range of conditions required to degrade all the different antigens. This problem might be circumvented by having isoforms of Ii with different sensitivities to proteolysis¹⁴, but the process would still be inefficient. The CPL may provide an environment in which peptide- and Ii-free class II are held in a receptive state (the 'floppy conformation'⁴) while antigens are proteolysed and loading occurs. As shown in the figure, occupied class II can exit at any time (F. Castellino and R. Germain, personal communication), allowing a complete sampling of antigenic peptides.

Like the transcytotic vesicles and specialized storage compartments of other cell types, the CPL seems to be relatively inaccessible to bulk endocytic content and cargo destined for conventional late endosomes/lysosomes^{1,3}. This provides the opportunity to control the range of antigens degraded. In the case of B cells, selective sorting and targeting of antigen internalized by mIg or Fc receptors to the CPL would increase the specificity and efficiency of antigen presentation. It is worth noting that the CPL of melanocytes, which are not conventional APCs, have greater access to bulk fluid phase

markers than the CPL of B cells².

Finally, how are class II and antigen targeted to, maintained in and recycled from CPL? The Ii is the most likely candidate to direct class II to the CPL, the sorting of the complex being directed by two dileucine motifs in the Ii cytoplasmic tail (ref. 15; L. Pond, personal communication). A dileucine motif has been implicated in sorting GLUT4 from the conventional endosomal pathway in adipocytes¹⁶. Targeting of antigen to the CPL is presumably also tightly controlled. The FcRII-B2 receptors used to deliver antigen to CPL in lymphocytes¹ also contain a dileucine motif¹³. Targeting of mIgG may be due to aggregation of liganded mIgG or to some signalling pathway: both mechanisms are used in other systems for sorting from early endosomes¹³.

Most class II in the CPL is not associated with Ii (refs 1, 2, 4), so how are peptide-free class II α/β molecules retained in the CPL? Studies *in vitro* have shown that unoccupied class II α/β aggregate, and that the aggregates dissolve upon addition of appropriate peptides¹⁷. In the CPL, such a mechanism could ensure that only loaded class II molecules are released to the cell surface. Alternatively, an accessory molecule may maintain class II in the CPL. The class II-like molecule, DM, which is essential for antigen presentation^{18,19}, is one candidate. In mutant cells in which DM is absent, surface class II molecules contain a peptide fragment of Ii (CLIP)²⁰, a phenotype which could result from premature release of class II from the CPL.

The immune system developed quite late in evolution, and is renowned for modifying existing pathways for its purposes rather than creating new ones. Identification of the CPL provides fresh insight into the extent that APCs have remodelled existing pathways to make class II presentable. □

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Will the alphas abscond?

Richard D. Petrasso

WHEN Princeton University's Tokamak Fusion Test Reactor (TFTR) began burning a 50:50 deuterium-tritium mix last December, a key question was what would become of the energetic alpha particles (helium nuclei) generated in the

(about 50 kG). In principle, these would be the heat source driving electric power generators.

When deuterium and tritium operations first started at TFTR, two scientific issues were paramount. First, would a large

fraction of the α s unexpectedly abscond from the plasma without depositing their kinetic energy into the plasma? And second, would the plasma be able to store and retain its thermal energy as effectively as it did when running in pure deuterium? At TFTR, because fusion power was found to scale as the square of the stored plasma energy content, any diminution in the plasma's ability to hold its thermal energy, the efficacy of which is measured by the energy confinement time τ_E , would be directly reflected in a low level of fusion reactions.

Encouragingly, the main message from the TFTR workshop was that unexpected losses of energetic alphas did not occur, and τ_E actually improved by 20 per cent to 0.18 s (R. Hawryluk, Princeton Plasma Physics Laboratory (PPPL)). At present, the reasons for the improvement are not understood, but are being actively investigated (K. Itoh, National Institute for Fusion Science).

Figure 1 shows the fusion power generated when three different mixes of deuterium and tritium ions were injected into the plasma. These beam ions, with an energy of about 100 keV, both heat and fuse with the background plasma ions. In the top trace, representing 30 MW of beam power injected for 0.75 s, the fusion power was seen to peak at the new record of 6.2 MW, 0.4 s after beam heating was initiated (J. Strachan, PPPL). About 75 per cent of this power results from the heating beams fusing with background ions and with energetic, unthermalized ions originating from the beams. After the peak, the fusion power then smoothly declined by 30 per cent over the remaining duration of the beam heating (0.35 s). For other discharges (lower two traces of Fig. 1), the fractional de-

cline was smaller (R. Budny, PPPL).

Compared with the proposed International Thermonuclear Experimental Reactor, ITER, the TFTR has a small plasma cross-section (minor radius 87 cm). So about 5 per cent of the α s, known as 'prompt losses', were expected to escape the plasma before depositing essentially any of their energy². (They move downwards, in this instance; the direction depends on the magnetic field and its gradient). The signal from these escaping α s closely tracks the total production of fusion power as determined from the measurement of 14.1-MeV neutrons (S. Zweben, PPPL). In contrast, fully confined α s in TFTR take about 0.2 s to deposit roughly 60 per cent of their kinetic energy into the plasma, about the same as the energy confinement time τ_E . Ideally, future reactors will operate so that the α energy deposition time is comparable to τ_E or smaller.

As well as these 'prompt-loss' α s, a similar fraction of α s is theoretically predicted to escape and hit the outer vacuum walls of the TFTR at slightly below the midplane (M. Redi, PPPL). Unlike the prompt losses, these escapees, known in the lexicon of the field as 'stochastic-toroidal-field-ripple losses'³, are expected to occur in large fusion reactors such as ITER (S. Putvinski, ITER team, San Diego). The loss occurs because of a sensitive interaction between small perturbations (~ 1 per cent) in the toroidal magnetic field (a consequence of the finite number of the toroidal magnetic field windings, 20 for the TFTR) and a class of α s for which the toroidal velocity component oscillates through zero⁴. Hints of these losses were obtained during the tritium experiments by particle detectors (S. Zweben, PPPL), but additional measurements and analysis are under way to assess whether these losses have the

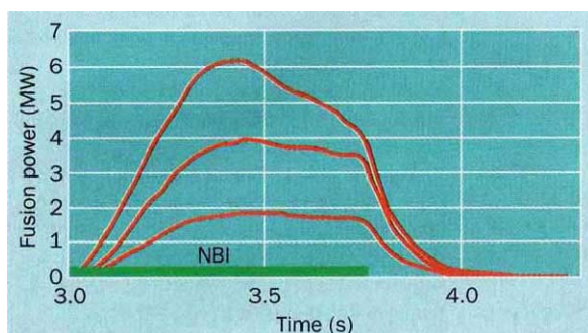


FIG. 1 Fusion power emitted from the burning of deuterium-tritium, for three discharges in which different proportions of tritium were injected via energetic heating beams into the plasma. Top trace, seven tritium sources (this set the record of 6.2 MW fusion power); middle, four sources; bottom, one source. NBI (neutral beam injection) indicates the duration of the heating beams. (From J. Strachan.)

fusion reaction. For a future reactor, losses of even a few per cent of the energetic alphas could have serious consequences. With losses of more than 15 per cent, the temperature needed for fusion ignition, about 250 million kelvin, would be difficult to sustain unless the thermal energy confinement was good. And the heat load of even 5 per cent of these particles could damage the vessel walls, especially if the heating was unevenly distributed over the surface. With the completion of an extensive sequence of deuterium-tritium (D-T) experiments, including one in which a record 6.2 MW of fusion power was achieved, an international workshop* gave researchers the chance to consider what had been learned.

As I described in a News and Views report three years ago¹, when the first such workshop was held, future fusion reactors are expected to run on a 50:50 D-T mix, as D-T fusion has a much higher reaction rate and requires a far lower temperature than other fuel combinations. The TFTR experiments are the first to try this mix, although the Joint European Torus (JET) ran two discharges using 13 per cent tritium in November 1991, setting the previous record of 1.8 MW fusion power. The end product of the D-T reaction is a 14.1-MeV neutron and a 3.5-MeV α . The neutrons, unlike the α s, are uncharged and therefore unconfined by the magnetic field of the tokamak

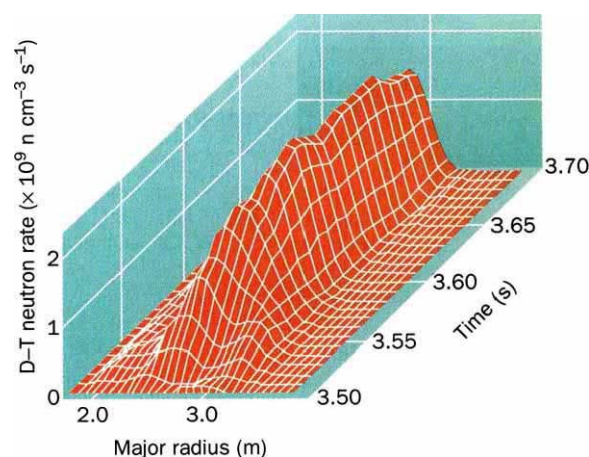


FIG. 2 Imaging of the 14.1-MeV neutrons produced in deuterium-tritium fusion allows mapping of the inflow and transport of trace amounts of tritium, injected at time $t = 3.50$ s into a deuterium plasma. The transport of both tritium and deuterium is crucial to sustaining the fusion burn. (From L. Johnson.)

* IEA US-Japan Workshop on D-T Experiments, Princeton, New Jersey, USA, 2-4 March 1994.

expected magnitude and scaling. For example, do they decrease with increasing plasma current? As this loss mechanism is directly germane to future reactors, it is especially important to check the theoretical predictions at TFTR.

An entirely different genre of α -loss mechanisms involves the interaction between coherent magnetohydrodynamic oscillations ('collective' oscillations) and the α s. Like Proteus, these oscillations can manifest themselves in different guises, as 'fishbones,' 'snakes' or 'sharkteeth'⁵, to name but a few, and if they grow large enough they can eject energetic particles, such as the heating beam ions or the α s, from the plasma (B. Coppi, Massachusetts Institute of Technology). At high concentrations (about 1 per cent), the α s themselves can drive these instabilities (unlikely in TFTR, but possible in future reactors).

The chosen operating parameters for TFTR kept these collective modes to a minimum, and for these levels there were no associated α losses (H. Hsuan, PPPL; and L. Zakharov, PPPL). But the discharge with maximum fusion power may have been affected — one school of thought attributes the droop in fusion power, at least in part, to a coherent mode that appeared just after the peak.

One collective oscillation that could be a particular problem in future machines is the α -driven Alfvén mode (H. Kimura, Japanese Atomic Energy Research Institute; C. T. Hsu, Massachusetts Institute of Technology). Amongst other conditions, this instability occurs when the average α velocity ($1.3 \times 10^7 \text{ m s}^{-1}$ at birth) is higher than the Alfvén velocity ($v_A = B/\sqrt{4\pi m_i n_i}$, where B is magnetic field and m_i , n_i the mass and number density of the ions). The good news is that it was not observed in the TFTR experiments (E. Fredrickson, PPPL; and R. Nazikian, PPPL), as indeed theoretical calculations had indicated. The ratio of α pressure (the product of number density, n_α , and kinetic energy, E_α) to magnetic field pressure ($B^2/8\pi \approx 100 \text{ atm}$) would need to be roughly 5 times greater than it typically was in the TFTR (C. Z. Chang, PPPL). But because the conditions are far more likely to be satisfied in future machines, efforts are being made to push the TFTR over the excitation threshold. One way that has been suggested is to drive a larger fraction of the plasma current off-axis (D. Spong, Oak Ridge National Laboratory).

And what about the α s that are well confined, which make up about 90 per cent of those born in the TFTR? As the α heating effects are only a few per cent, can we directly detect them through sensitive α -particle diagnostics? In fact, diagnostic methods are being developed that, for example, determine the density of the α s through their elastic collisions with plasma tritons. Some of these energy-boosted

tritons, known as knock-ons, then fuse with deuterons generating neutrons that have an energy up to 20.7 MeV. To detect these high-energy neutrons, and to avoid being swamped by the 14.1-MeV neutrons from the background D-T reaction, an energy threshold detector is to be used: based on a threshold nuclear reaction in beryllium, it is sensitive only to neutrons with energies above 16.5 MeV (R. Fisher, General Atomics, San Diego).

A further important element of the deuterium-tritium experiments has been the study of tritium transport itself. Rather than adding a high proportion of tritium, the essence of this experimental programme has been to inject trace quantities (~ 1 per cent) of tritium into deuterium plasma, either in the energetic heating beams (D. Jassby, PPPL), or by introducing neutral (cold) tritium gas at the plasma edge (P. Efthimion, PPPL).

In the latter case, the inflow and on-axis peaking of the tritons can be observed by imaging the emission of the 14.1-MeV neutrons produced in D-T fusion (Fig. 2). The indications are that trace tritium diffuses into the plasma core at about the same rate as trace helium. (Trace helium injections can assist our understanding of the transport of spent α s.) Both tritium and deuterium transport are known to be of great importance in sustaining the fusion power. In fact, it has been suggested that part of the decline in fusion rate (Fig. 1, top trace) may be due to an increased inflow of cold deuterium from the plasma edge (C. Skinner, PPPL), a process known as enhanced recycling.

And what happens to the 'ash' of spent α s? For a future machine in which α heating dominates over all other forms, might the ash accumulate in the plasma core, diluting the fusion fuel and quenching the fusion burn? Before the TFTR ceases operations this autumn, experiments currently under way should detect the small quantities of α ash within the machine, giving us our first glimpse of this transport (E. Synakowski, PPPL).

So what is this future machine? Given the TFTR's encouraging evidence that the α s did not abscond and that the energy confinement improved, the next logical scientific step is a device in which the α -heating is substantial or even dominant, and for which instabilities, either driven or possibly suppressed by the α s, can be readily studied. Will the α s then abscond, or instead heat the plasma? □

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Dilute pleasure

ADDICTIVE drugs owe their market not to the pleasure of using them, but to the pain of giving them up. Their withdrawal symptoms are so unpleasant that addicts will commit any crime to get more drug. So Daedalus wants to alleviate withdrawal. He recalls the traditional French alcoholic, who sips wine all day and much of the night. His blood-alcohol never drops to zero, so he never has a hangover. If cocaine and heroin could be sustained like this, instead of giving a brief rush of pleasure followed by hideous withdrawal pains, they would be less of a menace.

Many modern pharmaceuticals can be injected as a subcutaneous fatty 'depot', from which they leach out slowly into the patient's bloodstream. DREADCO's biochemists are now devising a blank, rechargeable depot. It will act as an internal buffer for addictive drugs. Most of them, such as amphetamines, cocaine and heroin, are nitrogen bases; they can combine reversibly with acids. So the new drug buffer is an inert fatty polymer with many acidic groupings. It will seize any drug that gets into the user's bloodstream, and slowly release it again. This equilibrium will need careful tuning. The buffer must absorb drug from the blood in levels much above the desired value, and feed it out again to stop the level dropping much below.

When perfected, DREADCO's 'Drugstat' will simply be injected into the addict — either as treatment or as part of the punishment for drug-related crime. His next dose will be a strangely muted experience. There will be no sudden rush of pleasure; as fast as he takes in the drug, the Drugstat depot inside him will seize it. The low equilibrium dose in his bloodstream will give him only a mild mental alleviation. Yet this alleviation will persist wonderfully. Drugstat will maintain the defined concentration in his blood, leaking out just enough drug to avoid withdrawal symptoms. Many days may pass before he has to repeat the dose.

Drugstat will calm the wild roller coaster of the addict's life. It will meter his drug so efficiently that he will need far less of it. His new mental stability may even let him hold down a job and buy his reduced dose without resorting to crime. Furthermore, the pallid, diluted pleasure of Drugstated addiction will no longer antagonize the respectable masses. Their puritanical envy and outrage at people who get pleasure without working for it, is the basic reason why drugs are illegal in the first place. Once Drugstat is in wide use, public opinion may at last permit drugs to be legalized, thus solving the whole problem. David Jones

Disposable cups have eco merit

SIR — In order to compare the relative merits of reusable and disposable cups I have examined their respective energy costs. First, I assembled a large energy database of the component technologies required to make various cup types. Wherever feasible, this information included all aspects of the energy costs of each technology from its initial extraction from the ground or harvesting stage to the final cup product. I then selected a median energy requirement for each cup technology from these data.

I collected a range of 8-ounce cups of each type from several countries and weighed them to establish a mass range for each type. I used the cup of median mass for each cup type, which closely matched the mean for each cup type, for a detailed energy assessment. Considering only the energy cost to manufacture the cups,

105.5 kJ per cup of electrical energy works out to a primary energy expenditure of 184 kJ per cup per wash.

Including the primary energy cost of washing into the cup comparison energy matrix increases the number of servings required from reusable cups, before their energy costs per use come down to the energy costs of the disposables, to 15 for the glass/paper cup pair, and 1,006 for the ceramic/polystyrene foam cup pair (see figure). These values are in agreement with those obtained in other studies using different methodologies (refs 3, 4). If one considers the lower UK or US generating efficiencies, the median primary energy values required to clean and sanitize a reusable cup rise to 300 and 278 kJ, respectively. Both these energy requirements are greater than the 198 kJ required to make a moulded polystyrene foam disposable cup.

To aid in the selection of appropriate cup types for different public service situations, I calculated the break-even servings required from the reusable cups to obtain a "required return rate" needed to equate in energy terms to the disposables. One serving per wash of the reusables in comparison to one serving before discard of the disposables required a return rate of 94–99.9%, using the Canadian generating efficiency. Thus, the most favourable comparison for reusables would require a return of more than 94 times out of a 100 uses (without loss or discard) for them to have lower energy consumption than the disposables. The least favourable comparison for reusable cups requires over 999 returns out of 1,000 uses.

Most people prefer to use a ceramic, glass or hard plastic cup than any of the disposable varieties. For everyday use in the home or in restaurants, where a reusable cup may last 500 or more cycles, their use makes sense. But my analysis demonstrates that, from the point of view of energy conservation, there is good reason to use one of the disposable cup types when the return rate is likely to be low. If a ceramic cup is used less than 39 times it would be energy-smart to use a paper cup. The equivalent comparison for the ceramic/polystyrene foam cups would require more than 1,000 uses of the former to consume less energy per use. Of course, two or more uses of either cup type before washing (for the reusables) or discard (for the disposables) decreases the energy cost per use proportionately.

Of the disposable cups used in this analysis, the moulded polystyrene foam cup consumes the least total energy

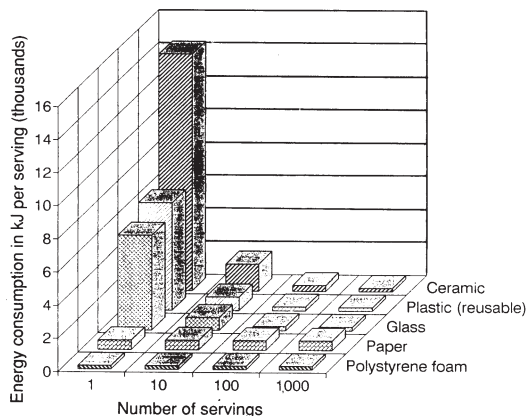
per unit. The uncoated paper cup, on the other hand, consumes the least fossil-fuel energy per unit, much of the energy for production being derived from the renewable sources of wood waste, lignin and the like.

A full version of this paper, including the databases, equations, sensitivity tests, and effects of recycle options, will be published shortly in *Environmental Management*.

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Energy consumption by reusable and disposable cups in kJ per serving. Assumes one use before washing for reusable cups, and one use before discard of disposable cups.

10–70 uses of a reusable cup are needed before the energy required per use comes down to the energy cost to make the disposable ones. (This comparison ignores the energy costs of cleaning and sanitizing required for commercial use of reusable cups.)

I collected information on hot water and operating energy requirements of commercial dishwashers. The electrical energy requirement range per cup per wash is 70–151 kJ. An energy-efficient under-counter dishwasher model gave a median value for cleaning energy cost of 105.5 kJ of electrical energy per cup per wash. To account for the primary energy consumption required to produce this electricity, the area of operation of the dishwasher has to be specified to account for the differing efficiencies of electricity generation in different countries, for example: Canada 57.3%; United Kingdom 35.2%; and the United States 38.0% (refs 1, 2). Taking the conservative Canadian generating efficiency as an example, the

HIV-1 infection of non-dividing cells

SIR — Onco-retroviruses can productively infect only actively dividing cells¹. In contrast, lentiviruses, including human immunodeficiency virus type 1 (HIV-1), can establish a productive infection in certain non-dividing cells^{2,3}. Of particular importance to HIV-1 replication *in vivo* is the infection of non-dividing, terminally differentiated cells of the monocyte/macrophage lineage such as microglia in the central nervous system⁴.

Bukrinsky *et al.* recently reported⁵ that a highly basic region of the HIV-1 matrix protein is responsible for the ability of HIV-1 to productively infect non-dividing cells (see Figs. 2 and 3 of ref. 5). This conclusion was based on the authors' observation that mutation of two adjacent lysine residues (matrix protein amino acids 25 and 26) to two threonine residues blocked infection of non-dividing cells by HIV-1. For these experiments, the authors used two types of non-dividing cells: γ -irradiated, CD4⁺ HeLa cells and aphidicolin-treated MT-4 T cells. Because they were able to direct bovine serum albumin, conjugated to the highly basic matrix protein domain, to the nucleus, they argued that this region of the HIV-1 matrix protein also targets the HIV-1 preintegration complex to the nucleus.

We have introduced a large number of mutations into the HIV-1 matrix protein, including the same two-amino-acid

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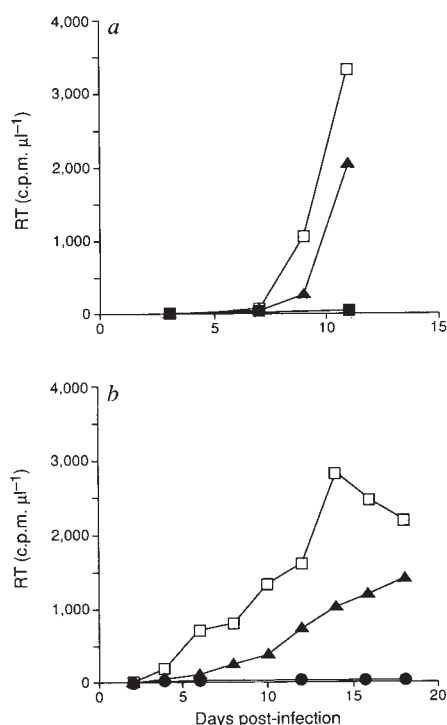


FIG. 1 Growth of the HIV-1 25KT/26KT MA mutant in the CEM(12D-7) T-cell line (a) and in primary human macrophages (b). The 25KT/26KT mutant was introduced into the infectious molecular clone pNL4-3 (open squares in a) for the T-cell line experiment, and into a macrophage-tropic derivative of pNL4-3 (pNL4-3(AD8); open squares in b) for the macrophage experiment. CEM(12D-7) cells were infected with equivalent amounts (normalized for reverse transcriptase activity; RT) of wild-type (open squares) or mutant-containing (closed triangles) NL4-3 virus. Closed squares, negative control (uninfected cells). Macrophages were infected with equivalent amounts of wild-type NL4-3(AD8), 25KT/26KT mutant-containing NL4-3(AD8) or NL4-3 virus.

Lys-Lys → Thr-Thr substitution reported in ref. 5. Following the transfer of this mutation (25KT/26KT) into the T-cell tropic molecular clone pNL4-3 and the macrophage tropic clone pNL4-3(AD8), we have analysed its effect on HIV-1 infection of both a T-cell line and terminally differentiated primary human monocyte-derived macrophages. The 25KT/26KT mutant virus efficiently establishes a productive infection in both dividing T cells (Fig. 1a) and non-dividing monocyte-derived macrophages (Fig. 1b). We observe slightly slower kinetics of virus spread in both the T-cell line and in macrophages, possibly due to an effect on the targeting of the Gag precursor to the plasma membrane⁶. As expected, the T-cell tropic HIV-1 isolate (HIV-1_{NL4-3}) does not productively infect the macrophages.

Although these results certainly do not prove that the region of the HIV-1 matrix protein described above is not involved in nuclear localization in some cell and virus

systems, they do indicate that the two basic residues studied by Bukrinsky *et al.* are not essential for productive infection of primary macrophages. Caution is needed in extrapolating results of HIV infectivity in irradiated HeLa cells or drug-treated T cells to primary human monocyte-derived macrophage cultures.

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EMERMAN *ET AL.* REPLY — As an extension of our earlier studies¹, we have examined the phenotypes conferred by amino-acid substitutions in HIV-1 matrix protein within the context of different molecular clones of HIV-1. A set of proviruses with mutations in either the nuclear localization signal (NLS)¹ in matrix (here called MA_{NLS}) or in the accessory gene, *vpr*, or in both the MA_{NLS} and Vpr were generated based on the LAI genome that encodes all the accessory proteins², including a full-length 96-amino-acid Vpr protein. The infectivity of these viruses was tested in non-dividing cells that were blocked in the cell cycle at G2 (Fig. 2).

In agreement with our previous studies, mutation of the MA_{NLS} interrupts HIV-1 infection of non-dividing cells. However, this effect is only observed in the context of a genome that contains a mutation in Vpr and is not observed in the presence of a full-length Vpr (Fig. 2). These results indicate that MA and Vpr contain redundant signals which govern infection of non-dividing cells and define, for the first time, a function for an accessory gene of HIV-1. Experiments that confirm the independent roles of HIV-1 matrix and Vpr proteins in nuclear import of viral nucleic acids and in virus infection of primary macrophages will be detailed elsewhere in a full paper³.

We believe that there is a simple explanation for the result of Freed and Martin. The clone used in our original study (HIV-1 MFD) is a IIb derived virus⁴, and like some isolates of IIb, contains a truncation of the Vpr open reading frame. In the case of MFD, a single nucleotide deletion truncates Vpr

by 55 amino acids. Thus, in that Vpr-negative background, MA_{NLS} mutants are unable to infect non-dividing cells. In Freed and Martin's experiment, the same matrix protein mutations introduced into an NL4.3 virus, attenuates but does not abolish HIV-1 replication in primary macrophages. Because the NL4.3 virus used by Freed and Martin contains a full-length Vpr gene⁴, the MA_{NLS} mutation is complemented by a functional Vpr protein. Thus Freed and Martin's result is exactly what one would predict if only one determinant (either MA_{NLS} or Vpr) were mutated. Although we agree with the result obtained in Freed and Martin's experi-

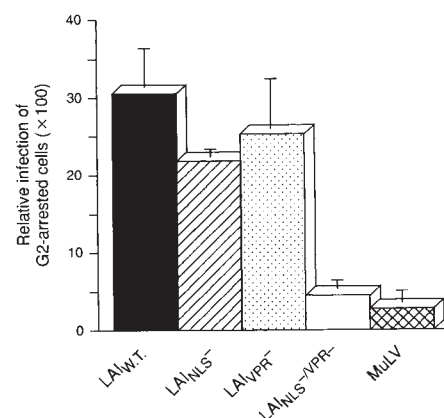


FIG. 2 Infection of non-dividing cells by wild-type mutant HIV-1. HeLa-CD4-LTR/ β -gal indicator cells were arrested in the cell-cycle at G2 by γ -irradiation as previously described⁵, and the ratio of infected cells in the non-dividing cultures relative to the number of infected cells in the untreated cultures were measured by counting the number of β -gal-positive cells⁶ (y-axis). The virus strains are based on the HIV-1 LAI genome². LAI_{W.T.} is the wild-type LAI clone, LAI_{NLS} contains a mutation of Lys 26, 27 to Thr in the matrix protein. LAI_{VPR} contains a frameshift mutation in the Vpr gene which would truncate the protein after 48 amino acids. LAI_{NLS-VPR} contains both of these mutations. MULV is an amphotropic MULV vector that contains the HIV-tat gene⁵. An MULV-based vector does not infect G2-arrested cells because cells must pass through mitosis for integration by onco-retroviruses^{7,8}.

ment, we disagree with their interpretation of that result. We conclude that the NLS within HIV-1 matrix protein is indeed a determinant in the ability of HIV-1 to infect non-dividing cells, including primary macrophages.

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Corrupted science?

Wilfred Beckerman

The Death of Economics. By Paul Ormerod. Faber: 1994. Pp. 230. £14.99.

THE worst thing about this book is its title. It ought to have been called "The Birth of Economics". The author asserts that the state of economics today is very much as it was in the Middle Ages, which may be true. In 1956, I published an article in *The Economic Journal* saying more or less the same thing, so I was naturally receptive to this part of Ormerod's message. But modern science was not in its death-throes in the Middle Ages; it was just getting going. The same applies to economics today. Many of Ormerod's very valid criticisms of most orthodox macro-economic theory originate, as he concedes, from within the economic profession itself. So economics is quite an active infant. Whether it will grow up properly, however, is another matter.

As Ormerod points out, economics was hijacked in the nineteenth century by a desire to emulate the mathematical approach that seemed to be so successful in the natural sciences. Consequently, promotion and prestige in the subject depend as much, if not more, on demonstrating technical mathematical ability rather than on genuine insight and imagination, let alone the familiarity with institutional constraints that characterized the work of the great earlier economists, such as Adam Smith, Ricardo and Malthus. Ormerod asserts, probably correctly, that there is an "internal culture in economics extolling esoteric irrelevance".

But Ormerod's evidence for his view is seriously flawed. For example, among the criticisms he makes of orthodox economics are: inadequate attention to the role of the share of profits in national income in explaining fluctuations in output and long-term growth; excessive reliance on bad simplifying assumptions; lack of appreciation of what goes on in the real world; excessive resort to mathematical hocus-pocus; and inadequate linkage of models of fluctuations with longer-run growth.

Yet, strangely enough, he completely overlooks the work of great economists such as Sir Roy Harrod or the Polish economist Michal Kalecki, who were certainly not guilty of any of these failings. He does refer, disparagingly, to one of Keynes's closest disciples, Richard Kahn, but persists in spelling his name as "Khan", which can be very misleading: readers may get him mixed up with many other Khans, such as Genghis Khan, the Aga Khan or Imran Khan.

Unlike Ormerod, Kalecki did not be-

lieve that economists really had much influence. Policy is determined by what the politicians want. Whatever policy they want to adopt — such as a deflationary policy in order to redress the balance of power in society and wrest it back from the trade unions — they will, as Kalecki warned back in 1960, find economists to provide theoretical justifications for their practical policies.

It is not the excessive mathematics and the adoption of absurd assumptions in order to make trivial problems mathematically soluble that will prevent the healthy development of economics. For it might be hoped that, as time goes by and economics grows up, every professional economist will have a good grasp of mathematics, so that kudos would go only — as in the natural sciences — to those with creative ideas. The wicked fairy's curse on economics at its birth was that economists would suffer the corrupting influence of trying to serve some political group or other, or their own ideological preferences. Natural scientists are less subject to this temptation, which is why the outlook for economics is not so bright.

When Ormerod discusses the weaknesses of micro-economics, he is again on rather weak ground. One of the main specific instances he provides of the weakness of orthodox economics is the issue

raised by environmentalists, namely the problem of the exhaustion of finite resources. In orthodox economics, as he explains, a shortage of any material would lead to a rise in its price, with all sorts of feedbacks that would tend to increase supply and reduce demand. And this is what has actually happened. The contrary predictions made in 1972 in *The Limits to Growth*, a ludicrous study which Ormerod generously praises, have been totally falsified by events.

The crowning inconsistency between Ormerod's general criticisms and his specific examples lies in his own attempt to escape from the constraints of what he calls orthodox economics. For, after spending five chapters criticizing modern macro-economic theory's excessive respect for mathematical modelling, our hero then makes a great bound and escapes by means of his own model, which miraculously works thanks to the special gadgets he has installed, including non-linearity in the equations, chaos theory, "attractor points" and so on.

However, despite the many defects in the details of the book, it is still an admirably lucid, well-written and informative read for the layman and even for those professional economists who ought to be more humble than they are. But the author displays a patchy knowledge of the literature and an inconsistency between his diagnosis and his own solution; and, by failing to spot the political element, the diagnosis is probably false to begin with anyway. □

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TAX-FREE bananas — eastern Caribbean islanders depend on bananas for half their income. Taken from *The Gaia Atlas of Planet Management* edited by Norman Myers. Now revised and extended, the atlas provides a wealth of information on environmental and political changes that affect our planet (Gaia Books, £16.99 (pbk)).

Genes are us

Mark Ridley

The Chosen Primate: Human Nature and Cultural Diversity. By Adam Kuper. *Harvard University Press*: 1994. Pp. 269. \$27.95, £22.25.

ANTHROPOLOGISTS often imagine themselves as engaged in a dialogue, or battle, variously implicit or explicit, between 'biological' and 'cultural' theories of human beings. Adam Kuper's new book is about this dialogue — and he does make it more of a civil dialogue than a battle. He is not concerned to argue for any new position, but has written an exposition of, and intelligent commentary on, the modern literature about it. Kuper edited, from 1985 to 1993, the journal *Current Anthropology*, which provides the best debating forum in the subject, and he has put his job there to good use in this book: it says something about the journal's importance that he can make more use of it than any other source, but without seeming to be biased or insular.

The Chosen Primate (no, I don't understand the title either) contains ten chapters, many of which are partly organized historically or personally. After an introduction, there is a chapter on human palaeontology, in which Kuper particularly shows how the evidence has forced us to accept more and more recent times of origin for distinctive human features, particularly the brain. He then has a chapter on the 'Washburn' school, which studied non-human primates and hunter-gatherers to understand human evolution by comparison. It is followed by one on the origin of culture, in which he argues that the cultural Rubicon was crossed in the transition to the Palaeolithic, and that the Neolithic transition was a lesser event. After a pair of chapters on eugenics and sociobiology, which I return to below, there are three more cultural chapters, on the family, sex and social structure respectively, and a final chapter where he looks into the future and suggests that we shall be able to adapt culturally to whatever population problems we create for ourselves.

The book has two admirable features. One is that it concentrates on big questions, and explains them accurately, briefly and intelligibly. It is also topical, so much so that some parts of it may even go out of date quickly, an unusual problem for a conceptual book in anthropology. The book ranges widely, across surprisingly large areas of (though by no means

all of) modern anthropology. It contains, for example, a paragraph or two that give a good idea of what 'world system theory' is, a few pages explaining where we are in the Freud-Westermarck debate about incest avoidance, another few pages about Freeman versus Mead on Samoa, and a delightful 10-page section about research on the !Kung bushmen. In each case, the material is easy to understand because it is made a part of a larger argument. The book is not one of those forgettable reviews of who said what, and what their main findings were.

The second admirable feature is the book's clarity and readability. One necessary skill for writers in a subject that blurs into philosophy is that they must confine their pen within the limits of their understanding. Kuper has this skill. He is not



The ethnographer Evans-Pritchard (1902–73) in the Sudan.

the sort of author to pull the wool over his reader's (or his own) eyes when he has nothing to say. Nor does he write to show off to impressionable apprentices, or the mutton-headed clerks that control the grant-giving quangos. Because I am about to turn to some more critical remarks, I must put on record the highest praise for a book on this subject: he does not (I think) use the word 'reductionist' even once.

The snag is the chapter on sociobiology. The fact that it is preceded by a chapter on eugenics and IQ testing arouses suspicion — suspicions Kuper does nothing to allay. He begins his sociobiological chapter by describing E. O. Wilson's *Sociobiology* as "a paean to genetics". The sociobiologists, indeed, "have been inspired by the great advances in human genetics". There

was, it seems, an early strong programme of sociobiology to discover single genes that code for complex behaviour and are expressed without learning (for he soon describes learning as an alternative to the sociobiologist's genes); this was crumbled by superior "critics", and gave way to a subtler system that allowed for learning and other complexities.

Now, there is much good material in the chapter, and some of the biologists he discusses, such as Lorenz and Tiger, may believe there are sociobiological imperatives to which our cultures have to conform. But its main tendency is, I believe, wrong. It consistently implies that adaptive explanations for human behaviour are (like the eugenicists and IQ hereditarists) making an embryological claim about human nature. Later on, sociobiology even collects a normative manifesto, when we find "feminist theorists" who "took a dim view of ethology and sociobiology" because those disciplines "assumed that a woman's destiny is to be played out as a wife and mother". But the logical structure of an adaptive argument implies nothing about biological imperatives that we culturally repress at our peril (these are in the realm of psychological introspection) or about 'constraints', in the folk sense of a limit, or about the way we ought to behave. Moreover, the 'genes' of adaptive arguments have little to do with those studied by real geneticists and are certainly not expressed independently of culture.

The 'genetic' assumptions of Darwinian arguments actually fit in well with the most liberated ideas about cultural diversity. They may even supply a defect in cultural theory. Our evolved mental apparatus, though neither a prison-house nor a moral standard, is the means by which we generate, or even reinvent, our culture. Kuper does not discuss the idea that the human mind is a *tabula rasa*, but it is notorious from work on artificial intelligence that no *tabula rasa* can learn anything, let alone invent a culture. An evolved, adaptive learning program, however, can.

Kuper's book is an excellent introduction to an eternally awkward, though fascinating, area of anthropology. He does sometimes — rather overdoing the dichotomy, to my taste — talk about something called "the biological party". If there are to be parties, I wish he had found room for a third: the party whose slogan is "a plague on both your houses". □

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Ballooning fruitfly literature

Peter A. Lawrence

The Development of *Drosophila melanogaster*. Two-volume set. Edited by Michael Bate and Alfonso Martinez Arias. Cold Spring Harbor Laboratory Press: 1994. Pp. 1,558. \$350.

ON my shelf, some of the time, there is a tattered old copy of Milislav Demerec's edited volume *The Biology of Drosophila*. The book, published in 1950, covers what was known at the time about the anatomy and development of the fruitfly. Some of the chapters are the result of years of full-time work by one author and the whole is an indispensable source, so much so that the pages need, even now, to be constantly repaired with tape and the binding stitched up.

Now we have the new multi-authored *The Development of Drosophila melanogaster*. This also promises to be useful. Once again, most things are covered; oogenesis, spermatogenesis, the laying down of the embryo's body plan, the adult and larval epidermis, the trachea, the mesoderm, the gut, metamorphosis, neurogenesis and the acquisition and modification of the central nervous system, plus a good chunk on the visual system. As usual with such books, the whole is not much more than a sum of the parts — the authors write individually, independently; their contributions, even though they cover much of the known ground, do not link together to guide the reader, and things get missed out. For example, there is nothing on sex determination and the coverage of the most interesting genetic system known anywhere is scanty — I mean, of course, the bithorax complex. It is not a book where big ideas are given emphasis; rather they are embedded in a mass of facts, factlets, guesses, speculations and rumours.

Few modern scientists, I think, would spend years producing a chapter for a book; most now prefer to feed the insatiable maws of the secondary literature, or to write papers and grant applications. So editors producing a multi-authored work need disciplinary qualities: they need to have a clear vision of what is contemplated, the clarity to transmit this vision to the authors and the resolution to insist that the authors conform. In their preface, the editors of the new work declare a worthy purpose: they wish to update and to replace Demerec. If so, then they need to cajole the best to write for them and to insist that those people give us the meat and throw away the ephemera; there are plenty of other places to seek out current opinion. Indeed, the editors have per-

suaded many luminaries to write on their own subjects, but some have grasped the opportunity too eagerly. They have described every little gene, every matter and measurement, every unpublished experiment — like conservationists on a treasured landscape that is threatened with a new motorway.

In guiding and curbing their contributors, the editors therefore seem to me to have been far too indulgent, and the outcome is an elephantine book that in places exhausts as much as it informs. One way to save words would have been to curtail the practice of quoting one's own unpublished experiments and those of friends; it amounts to the propagation of rumour and usually means no more than: "please remember folks, I think I did this

too!" Such statements do not help the reader one jot and do not make the evidence any more convincing. Nevertheless, many chapters are excellent and fulfil exactly the stated purpose: examples are chapters on the anatomy and development of the mesoderm by Michael Bate, on the pole plasm by Daniel St Johnston and on the retina by Tanya Wolff and Donald Ready. Such articles make the book of real value to the specialist.

In order to succeed, books, like balloons, need the right mix of hot air and ballast. This one has plenty of both. We shall have to see if it can fly for long. □

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A human geneticist's odyssey

Arno G. Motulsky

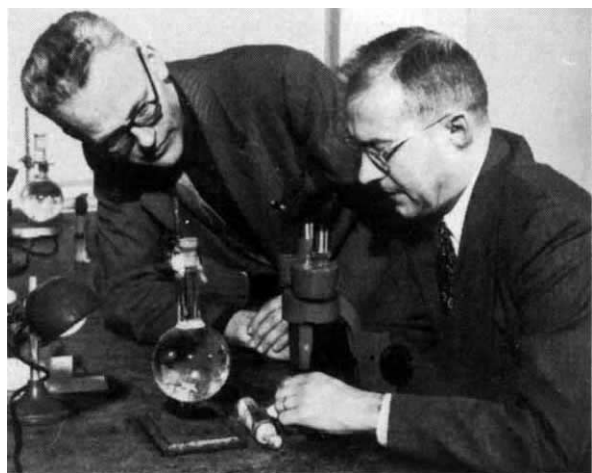
Physician to the Gene Pool: Genetic Lessons and Other Stories. By James V. Neel. Wiley: 1994. Pp. 460. \$24.95, £18.95.

JAMES NEEL is an American pioneer of human and medical genetics. In this book, he gives a personal account of his research, describes modern understanding of genes and passionately assesses the principal genetic problems facing humanity.

Neel obtained his PhD at the University of Rochester under Curt Stern, the eminent *Drosophila* geneticist who later wrote an influential textbook of human genetics. After being exposed to many luminaries in US genetics, Neel decided to cast his lot with human genetics and obtained medical and hospital training. This was a courageous decision in the early 1940s when reputable American geneticists, aware of earlier eugenic excesses and the Nazi abuses in Germany, saw human genetics as a soft and tainted science.

A young Army medical officer after the Second World War, Neel was responsible for follow-up studies of atomic bomb survivors in Hiroshima and Nagasaki and has persisted with this work ever since. The results of extensive genetic studies of children of survivors conceived after the bombings are not as well known as they should be. No statistically significant medical or biological differences were detected between subjects and controls (including chromosome aberrations and

protein mutations), although there were some slight untoward effects on pregnancy outcomes and on mortality. On the reasonable assumption that some mutations must have been induced, the radiation dose that would double the background level of mutations in humans was calculated from these data, and from parental exposure, as between 1.7 to 2.3 sieverts (170 to 230 rems) for acute radiation exposure, and was inferred to be



James Neel (right) with W. R. Spencer in 1954.

about 4 sieverts (400 rems) for chronic radiation exposure. So humans do not seem to be particularly sensitive to radiation-induced mutation. In mice, where more extensive data are available, Lewis and Neel calculated a similar doubling dose. This is the most comprehensive human study of the genetic effects of radiation and is a key resource when assessing radiation accidents such as that at Chernobyl.

A frequent participant in committees on radiation risk assessment, Neel believes that current radiation exposure

From Physician to the Gene Pool

standards are conservative but appropriate. He expresses concern, however, about the distorted perspectives and the regulatory overkill of US government agencies regarding radioactive waste deposits. Many millions or even billions of dollars may be spent to avert a single radiation-related 'health effect' while society neglects less expensive public measures that would be of much greater benefit to health.

W. J. Schull — a statistical geneticist — and Neel studied the effect on child health of the relatively frequent consanguineous matings in Japan. They found a small but definite effect on the frequency of physical defects and mortality as well as on stature, psychometric measurements and development of offspring, indicating the role of so far undefined recessive genes. Schull wrote a charming nontechnical view of his impressions of Japan (*Song Among the Ruins*, Harvard University Press, 1990).

In the 1960s, Neel and a multidisciplinary team ventured repeatedly into the rainforest of Brazil to study human evolution. They investigated the Yanomami, a remote Indian tribe that in Neel's judgement came close to having the lifestyle of early humans. Of adult males, 25 per cent were killed in violent encounters, infanticide was practised for birth defects and for 20–25 per cent of female births, and inbreeding was intense. There were marked genetic differences between Yanomami villages, a striking example of Sewell Wright's evolutionary model of random drift. The headmen in each village had more wives than other men and twice as many children. Neel thinks that genes may be involved in achieving headman status.

Assessing the future, Neel deplores the increasing political power of the aged in the United States and assigns a low priority to research into ageing that might lead to an extension of the human lifespan. His overriding concern, however, is global overpopulation and the prospect of epidemics, malnutrition and an impoverished ecosystem. He believes that all countries should treat this issue as the "social equivalent of a military emergency" and strive to achieve an egalitarian worldwide two-child policy.

His recommendations for eugenics (optimization of genotype expression) as well as wide use of genetic screening and counselling are in agreement with the opinions of most human geneticists, but his assessment of today's research trends is more controversial. He rejects current emphasis on gene therapy for genetic disease, which he feels requires more long-term assessment and is inappropriately popular because it uses high technology, does not challenge religious beliefs and does not require self-discipline. Similarly, he downgrades the Human Genome

Project as contributing little to the solution of the main genetic problems facing humankind. He believes that our concern with molecular genetics is diverting attention from the important problems affecting the human gene pool. Before the First World War, geneticists knew little about genes but worried about the future, whereas today's geneticists know much more about genes but largely ignore the broad issues facing humankind. Human geneticists are becoming mere "gene mechanics" and "DNA jockeys".

Neel has led an interesting life. He writes and explains well and shares many interesting and amusing episodes with the

reader. Although there will be disagreement with some of his judgements, his arguments command attention. Here is a highly respected human geneticist giving a somewhat different view of human genetics from that usually presented in the scientific and public media. The book is strongly recommended to geneticists, biologists and medical professionals as well as the intelligent public and policy-makers. □

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Preparing the ground for airwaves

Willem Hackmann

The Early History of Radio from Faraday to Marconi. By G. R. M. Garratt. *Institution of Electrical Engineers/Science Museum, London: 1994. Pp. 93. £19.*

GERALD Garratt, who died in 1989, spent most of his career at the Science Museum in London, where he was for many years in charge of the communications collections. This slim posthumous volume is based on his interest in the early history of telecommunications. Seven brief chapters review in chronological order the parts played by Michael Faraday, James Clerk Maxwell, Heinrich Hertz, Oliver Lodge, Aleksander Stepanovich Popov and Guglielmo Marconi in preparing the ground for radio broadcasting. The final chapter on Marconi was prepared, after the author's death, by his daughter, Susan, and is based on papers published by the Institution of Electrical Engineers and elsewhere in the early 1970s. The work of Joseph Henry, David Edward Hughes and George Francis FitzGerald is also dealt with in passing.

The author starts with the genesis of the electromagnetic theory beginning with Hans Christian Oersted's epochal experiment of 1820. This turned Newtonian science on its head, for Oersted demonstrated not only the relationship between electricity and magnetism, but also that there were forces that appeared to operate in circles. André-Marie Ampère gave these discoveries their mathematical foundation, but this did not satisfy a natural philosopher of the stature of Faraday. It was precisely the consequences of Ampère's mathematical ideas on the physical nature of matter that started Faraday on his quest. On 24 June 1837, Sir William Bragg, the then president of the Royal Society, broke the seal of a document Faraday had deposited there on 12 March 1832. In this fascinating manuscript Faraday refers to wave propagation,

which he saw as a consequence of his concept of lines of force in motion of a magnetic field. Maxwell produced his mathematical synthesis in the 1860s, although, as the author points out, the often stated claim that Maxwell predicted the existence of radio waves is at most a half-truth. FitzGerald, however, was convinced of their existence, but it was Hertz who confirmed this experimentally in 1887–88.

Lodge, as Henry before him, demonstrated the oscillatory nature of the Leyden-jar discharge. In the case of Lodge, this was in the context of improving the lightning conductor, but his investigations also prepared the ground for Marconi. One of the author's objectives was to demonstrate how essential Lodge was to the development of radio. Far in advance of any other, he appreciated the need for resonance between transmitter and receiver. The chapter on Marconi deals only with his introduction to the scientific fraternity in England in 1896, his relationship with Sir William H. Reece, chief engineer to the Post Office, and the successful English Channel trials of May 1897. These started the Post Office's interest in wireless telegraphy.

So what of Popov? The author agrees with the harsh judgement made by Charles Susskind in 1962, that at the time Marconi demonstrated his system, Popov did not possess a practicable method of radio-communication.

This is not intended to be an original book; most of the conclusions are well known. Nor does it deal with more recent scholarship. But it is a very readable account, most useful to non-historians who want a brief survey of the subject, and to students. □

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Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes

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Endocytosis of antigen by antigen-presenting cells results in the production of peptides that bind to newly synthesized class II molecules of the major histocompatibility complex. A new population of class II-enriched vesicles has been discovered in B lymphocytes that accumulate internalized antigen but are distinct from endosomes and lysosomes. These vesicles also transiently accumulate newly synthesized class II and class II-peptide complexes and appear to be a compartment specialized for the transport and loading of class II molecules.

MAJOR histocompatibility complex (MHC) class II molecules exit the endoplasmic reticulum (ER) as nonamers of three invariant chain (Ii) polypeptides and three α/β heterodimers¹, but are found on the plasma membrane as α/β heterodimers associated with antigenic peptides². The peptides are derived from extracellular antigens internalized by endocytosis or from endogenous proteins with access to the endocytic pathway³⁻⁵. Conversion of $\alpha/\beta/Ii$ complexes to peptide-loaded α/β dimers occurs during the transport of newly synthesized MHC class II molecules, presumably during a characteristic lag between the exit of class II molecules from the *trans*-Golgi network and their subsequent appearance on the cell surface⁶⁻⁸. These events probably occur in endocytic organelles: for example, Ii chain diverts newly synthesized class II molecules to endosomes^{9,10}, presumably by direct transport from the *trans*-Golgi network¹¹. Ii chain is then cleaved, rendering α/β dimers competent to bind immunogenic peptides^{12,13}. Precisely where these events occur is uncertain, but early endosomes, late endosomes and lysosomes have all been implicated in various cell types¹⁴⁻¹⁶.

Although several endocytic compartments each play some role in antigen processing, professional antigen-presenting cells (APCs) may have specializations that complicate interpretation using concepts defined for non-presenting cells. APCs such as Langerhans or dendritic cells contain distinct Birbeck granules that contain class II molecules^{10,15}. Similarly, human B lymphoblasts were found by immunocytochemistry to have class II-containing vesicles that were possibly distinct from endosomes and lysosomes¹¹. B cells represent an interesting example as they are potent APCs but, paradoxically, exhibit low rates of fluid endocytosis. At low antigen concentrations, B cells rely on binding to specific membrane immunoglobulins to internalize sufficient amounts of antigen for presentation to T cells^{17,18}.

From a cell biology point of view, an antigen-processing compartment should have characteristics common to both early and late endosomes. Although late endosomes have a more acidic internal pH and a high concentration of proteases which may favour the generation of antigenic peptides, they are relatively inefficient at recycling molecules back to the plasma membrane. Early endosomes mediate efficient recycling, but are relatively poor in proteases. In addition, transit through early endosomes is very rapid (1–2 min)¹⁹, which is a problem given that peptide binding to class II molecules is relatively slow²⁰. Thus, endosomes or lysosomes in APCs may be specifically modified to

facilitate peptide production and/or binding to MHC class II molecules.

A new MHC class II compartment in A20 B cells

To identify organelles involved in class II transport, B cells were fractionated by free flow electrophoresis (FFE), a technique used for other cell types to isolate early endosomes, late endosomes and lysosomes^{19,21}. We used a derivative of the A20 B-cell lymphoma transfected with an internalization-competent form of the mouse Fc receptor (FcRII-B2); this facilitated selective labelling of endocytic compartments and delivery of antigen for presentation²². A20 cells also have a small intracellular pool of MHC class II molecules as determined by immunofluorescence, immuno-electron microscopy and cell-surface biotinylation (data available on request). In contrast to B-cell populations with large intracellular pools of class II molecules²³, A20 cells degrade little newly synthesized class II, which instead is efficiently transported to the plasma membrane (see later). Thus, most of the α/β dimers synthesized in A20 cells were potentially functional.

Fractionation of A20 cells by FFE proved to be similar to fibroblasts, hepatocytes and MDCK cells^{19,21,24,25}. Typically, an endosome-enriched, cytosol-free membrane fraction containing >80% of endosomal, Golgi, and plasma membrane markers and partially depleted in the ER marker β -glucosidase, was first prepared by flotation in a discontinuous sucrose density gradient¹⁹. This fraction also contained ~90% of the total MHC class II, as determined by quantitative western blotting. When these or crude membranes were separated by FFE, a major 'unshifted' peak of cell protein was observed that co-migrated with markers of ER and plasma membrane (for example, ¹²⁵I-labelled IgG-immune complexes bound at 0 °C) (Fig. 1a). A marker of the Golgi complex, mannosidase II, migrated as an overlapping peak that was shifted towards the anode. The main peak of lysosomal enzyme activity (β -hexosaminidase) was shifted even further towards the anode, but was slightly broader than that seen in fibroblasts¹⁹.

Endocytic organelles in A20 cells fractionated as expected. To label early endosomes and recycling vesicles, cells were incubated with horseradish peroxidase (HRP)-labelled immune complexes (ICs) or ¹²⁵I-labelled transferrin (Tfn) before homogenization; late endosomes and lysosomes were labelled by allowing the cells to internalize Fc receptor-bound ¹²⁵I-ICs (Fig. 1 legend). As

shown in Fig. 1*b*, early and late endosomes migrated as overlapping but distinguishable peaks which were not as anodally deflected as the lysosomal marker β -hexosaminidase. Transferrin-containing early endosomes and recycling vesicles migrated at the same position as early endosomes labelled by HRP-ICs internalized for 15 min, but as a peak broader than that found for fibroblasts^{19,25}.

FFE fractions were probed by western blot with a polyclonal antibody against α - and β -chain (I-A^d). As expected, most of the class II molecules were in the unshifted peak, reflecting the presence of α/β dimers on the plasma membrane (Fig. 1*c*). Surprisingly, a second peak of class II gave a unique migration pattern, being deflected even more anodally than lysosomes. Although the second peak accounted for only a small percentage

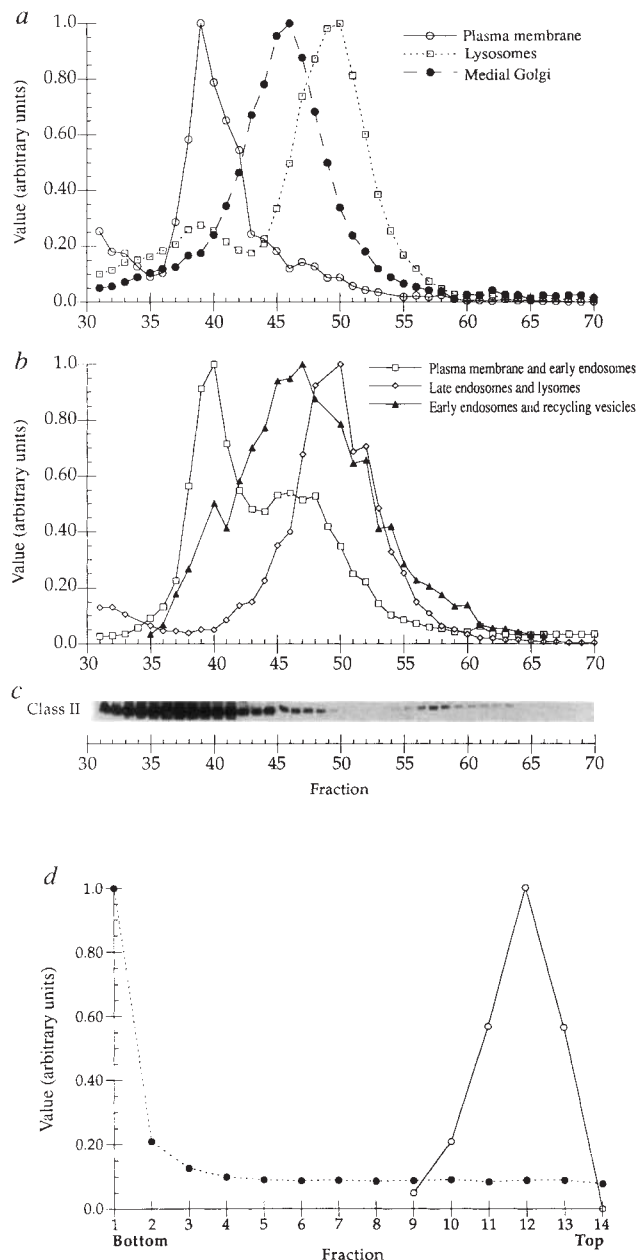
of the α - or β -chains in the starting material, it did not represent a subpopulation of plasma membranes. Markers of cell-surface proteins (¹²⁵I-ICs bound at 0 °C; ¹²⁵I-labelled plasma membrane proteins) were not found in this region of the FFE profile (ref. 21, and data not shown). A small fraction of endosomal and lysosomal markers in the anodally deflected region did not form a distinct peak, the main peaks of endosomes and lysosomes being well removed from the second peak of class II.

As the anodally shifted peak of α - and β -chains was resolved from all other known markers, we designated it a distinct population of class II-containing vesicles (CIIV). The CIIV fractions must have contained membrane vesicles because they could be collected by centrifugation at 20,000g. Moreover, if these membranes were collected on filters and hypotonically disrupted, they

FIG. 1 Fractionation of MHC class II-containing compartments in A20 cells by free flow electrophoresis (FFE). *a*, Separation of endogenous markers. To provide a marker for plasma membrane, A20 cells were incubated on ice (2 h) with ¹²⁵I-labelled DNP-BSA/anti-DNP IgG immune complexes (ICs). The cells were then homogenized and an endosome-enriched membrane fraction separated by FFE. Fractions assayed for ¹²⁵I-ICs (plasma membrane, open circles), β -hexosaminidase (lysosomes, squares), and mannosidase II (medial Golgi, filled circles). Cathode, left; anode, right. Activities are given in arbitrary units. *b*, Separation of early and late endosomes. Early endosomes were labelled by pre-binding HRP/anti-HRP ICs on ice and then warming to 37 °C for 15 min; under these conditions, ~50% of the ligand was internalized to early endosomes, with the rest remaining on the plasma membrane (squares). Early endosomes and recycling vesicles were labelled by allowing A20 cells to internalize ¹²⁵I-Tfn (60 min, 37 °C) (filled triangles). Late endosomes and lysosomes were labelled by incubation for 2 h at 37 °C in the presence of ¹²⁵I-ICs (diamonds). Labelled cells were homogenized and fractionated by FFE. *c*, Identification of MHC class II-containing compartments by western blot. A20 cells were fractionated by FFE and the presence of MHC class II in each fraction determined by western blot with a polyclonal anti-class II antibody. The distribution of the α -chain is shown co-aligned with the FFE marker profile and clearly shows a peak of class II-containing vesicles (CIIV) with a markedly anodal migration. *d*, Percoll-gradient fractionation of the anodally shifted CIIV fraction. FFE fractions containing CIIV and lysosomes were pooled and centrifuged on a Percoll density gradient. The CIIV (class II, open circles) and lysosomes (β -hexosaminidase, filled circles) were completely separated from each other, with the lysosomes sedimenting near the bottom of the gradient (left); class II molecules were detected by western blot only in low-density fractions (right).

METHODS. *a*, For FFE, a slight modification of established procedures was used²¹. Cells were suspended in cold TEA buffer containing 10 mM triethanolamine, 1 mM EDTA, 10 mM acetic acid, pH 7.4, 250 mM sucrose at 3×10^7 cells per ml and passed 8 times through a ball-bearing homogenizer (gap, 28 μ m). The homogenate was treated with 100 μ g ml⁻¹ DNase I (10 min, 25 °C) and spun for 10 min at 250g. Total microsome (100,000g pellet) or a crude endosome-enriched membrane fractions were prepared to concentrate the material being applied to the FFE chamber¹⁹. For endosome-enriched fractions, the low-speed supernatant was brought to 1.4 M, overlaid with 2 layers of TEA containing 1.25 M and 0.25 M sucrose, and centrifuged for 1.5 h at 35,000 r.p.m. in a Beckman SW-41 Ti rotor. Low-density membranes (0.25 M/1.25 M interface) and high-density membranes (1.25 M/1.40 M interface) were collected; the former contained 90% of the class II, endosomal, plasma membrane and Golgi markers. After diluting to 0.25 M sucrose, membranes were briefly treated with trypsin (2 μ g per mg protein for 10 min at 37 °C; stopped by addition of 20 μ g soybean trypsin inhibitor (SBTI) mg⁻¹). This treatment does not affect ATP-dependent endosome acidification or the cytoplasmic domains of various membrane proteins, including Ii chain^{19,21,24,25,33,34}.

The sample was applied to a Bender and Hobein Elphor VaP 21/22 FFE chamber at a rate of 100 μ l min⁻¹ at 250 ml h⁻¹ and 130 mA. Marker enzyme and protein assays have been described^{19,25}. MHC class II distribution was determined by western blot using a polyclonal antibody against affinity-purified I-A^d (gift from R. Kubo) and visualized using enhanced chemiluminescence (ECL, Amersham). *b*, Endocytic compartments in FcRII-B2-transfected A20 cells were selectively labelled as described in the text using ¹²⁵I-labelled DNP/anti-DNP ICs (DNP, dinitrophenyl), HRP-labelled ICs (5 μ g ml⁻¹ HRP and 20 μ g ml⁻¹ affinity-purified rabbit anti-HRP), and/or ¹²⁵I-Tfn (5 μ g ml⁻¹). HRP distribution was determined by incubating 100 μ l of



each FFE fraction with 100 μ l TEA buffer containing 1 mg ml⁻¹ OPD (o-phenylene diamine), 1% NP-40 and 0.03% H₂O₂ for 1 h at 25 °C. The reaction was stopped by the addition of 10 μ l 6 M HCl and the absorbance at 492 nm was measured. *d*, FFE fractions were pooled, made to 25% Percoll in TEA, and centrifuged for 30 min at 25,000g in a Beckman TLA-100.3 rotor.

could present peptide antigens to T-cell hybridomas (not shown). Class II molecules were enriched >100-fold in CIIV fractions compared to total protein. This enrichment was also evidenced by electron microscopy (see below) and by the fact that CIIV contained <1% of markers for the Golgi, ER or plasma membrane and <10% of endosomal or lysosomal markers.

CIIV are not a subpopulation of lysosomes: after centrifugation of CIIV fractions in Percoll, all α - and β -chains were recovered in the low-density regions of the gradient, well away from the contaminating lysosomes marked by β -hexosaminidase (Fig. 1d). Indeed, centrifugation of whole homogenates of A20 cells under various conditions indicates that these cells have no detectable MHC class II in heavy-density membranes; class II is only found in membranes of a density similar to early endosomes (our unpublished results, and R. Mitchell, personal communication).

CIIV are part of the endocytic pathway

Although principal peaks of early and late endosomal markers on FFE density gradients were distinct from CIIV, they did partially overlap. To determine whether endocytic tracers gained access to CIIV or simply represented endosomal contamination of the CIIV fraction, we used immuno-electron microscopy to examine CIIV isolated from cells that had internalized Fc receptor-bound antigen. The CIIV fraction consisted of a population of vesicles, most of which stained heavily for MHC class II (Fig. 2b). Although somewhat heterogeneous in size and shape, the vesicles were typically electron-dense and contained membrane infoldings that were also class II-positive. Similar vesicles were observed in frozen sections of intact cells, although infrequently (1–2 per cell cross-section), consistent with the small internal pool of class II in A20 cells. Either after FFE or in intact cells, CIIV morphologically resembled MHC class II-positive

compartment (designated MIIC) vesicles seen in human lymphoblasts¹¹. CIIV structures were rarely observed in non-shifted FFE fractions, where the major class II-positive membranes were sheets and vesicles characteristic of plasma membrane (Fig. 2a). No class II-positive plasma membranes or other morphologically identifiable organelles were found in CIIV fractions. Thus, CIIV represented an enriched population of class II-containing vesicles.

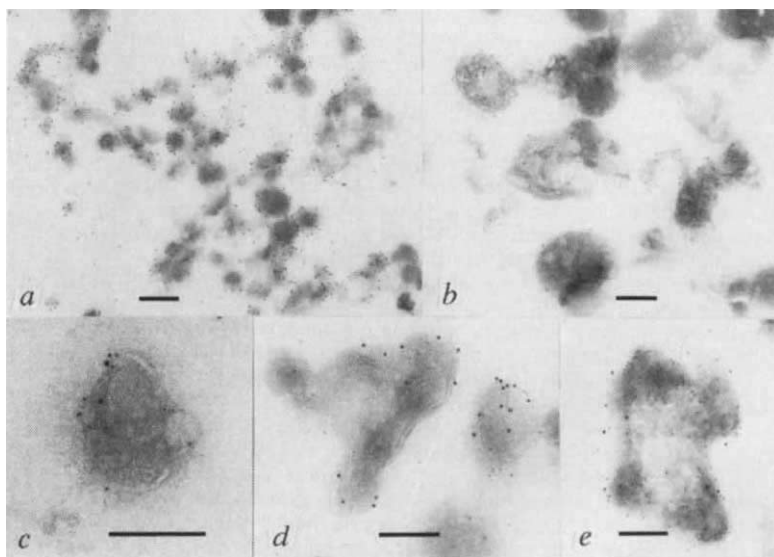
The CIIV isolated from cells incubated with HRP-anti-HRP immune complexes were found to contain immunoreactive HRP (Fig. 2d, e). Although there was some variation in the degree of anti-HRP labelling among the vesicles, most class II-positive structures were also positive for HRP. CIIV must therefore be connected with the endocytic pathway. Consistent with the fact that most of the internalized complexes did not reach CIIV but instead were derived to endosomes and lysosomes (Fig. 1b), abundant HRP-positive vesicles were found in endosome-lysosome-containing FFE fractions, but these were generally negative for class II (not shown). Thus, CIIV must represent a distinct subpopulation of endocytic vesicles that receive a fraction of internalized extracellular antigen under conditions known to correlate with antigen presentation in A20 cells²².

Class II-positive CIIV also contained transferrin receptor (Tfn-R) (Fig. 2c). Although only a small fraction of the transferrin internalized by A20 cells reached CIIV (Fig. 1b), CIIV contained at least one marker of the receptor-recycling pathway, supporting the idea that CIIV might recycle their contents back to the plasma membrane and distinguishing CIIV from MIIC, which are transferrin-negative¹¹.

CIIV and α/β dimer transport

Most plasma membrane proteins reach the cell surface within 30 min of synthesis, but in human lymphoblasts the appearance

FIG. 2 Characterization of isolated CIIV as MHC class II-positive, endocytic vesicles. **a**, Class II-positive plasma membrane in the unshifted fraction. A20 cells were fractionated by FFE (as for Fig. 1). Membranes contained in the pooled unshifted fractions were fixed, collected by centrifugation, cryosectioned and labelled with rabbit anti-MHC class II and 5-nm gold-labelled protein A. As expected, abundant and strongly class II-positive membrane vesicles and sheets corresponding to plasma membrane were seen. **b**, CIIV fractions are enriched in a population of class II-positive vesicles. Purified CIIV were isolated from A20 cells as for Fig. 1, fixed and collected by centrifugation. The pelleted CIIV were cryosectioned and stained for MHC class II as in **a**. The fraction contained a population of vesicles with characteristic membrane infoldings. Most of the vesicles were positive for MHC class II; both the limiting and internal membranes were labelled. Although a few class II-negative structures were evident, other morphologically identifiable structures or plasma-membrane-derived vesicles were not found in this fraction. **c**, CIIV contain transferrin receptor. Cryosections from isolated CIIV fractions were double-stained for MHC class II (5-nm, small gold) and for Tfn receptor (10-nm, large gold). **d**, **e**, CIIV contain internalized horseradish peroxidase. CIIV were isolated from A20 cells which were labelled with HRP-immune complexes for 1 h at 37 °C, conditions sufficient to prime antigen presentation by these cells²². The CIIV were fixed sectioned and stained for class II (large gold) as well as for HRP (small gold). Most of the class II-positive vesicles characteristic of CIIV were also positive for HRP, but there was some variability in the relative extents of labelling. Some structures contained relatively small amounts of immunoreactive HRP (**d**), whereas others were heavily labelled for both HRP and class II (**e**). Importantly, there was no HRP staining when CIIV were used that had been isolated from cells not exposed to the immune complexes before fractionation. Scale bars in **a**–**e**, 0.2 μ m. **METHODS**. A20 B-lymphocytes were labelled with HRP-ICs for 1 h at 37 °C and then fractionated by FFE as for Fig. 1. Isolated CIIV were fixed with 1% glutaraldehyde in TEA buffer containing 1% BSA and



pelleted by centrifugation for 3 h at 35,000 r.p.m. in a Beckman SW-41Ti rotor at 4 °C. The pellets were embedded in 10% gelatin and cryo-protected in 2.1 M sucrose before freezing and sectioning. Contrasted sections were prepared using 2% uranyl acetate in methyl cellulose. Single and double labelling and preparation of protein-A gold was accomplished using conventional procedures. MHC class II was visualized using antiserum from a rabbit immunized with affinity-purified I-A^d; Tfn receptor was visualized using an autoreactive mouse monoclonal antibody against a conserved region of the receptor's cytoplasmic domain (gift from I. Trowbridge); HRP was visualized using an affinity-purified rabbit anti-HRP antibody (Boehringer Mannheim). Sections were viewed with a Philips 401 electron microscope.

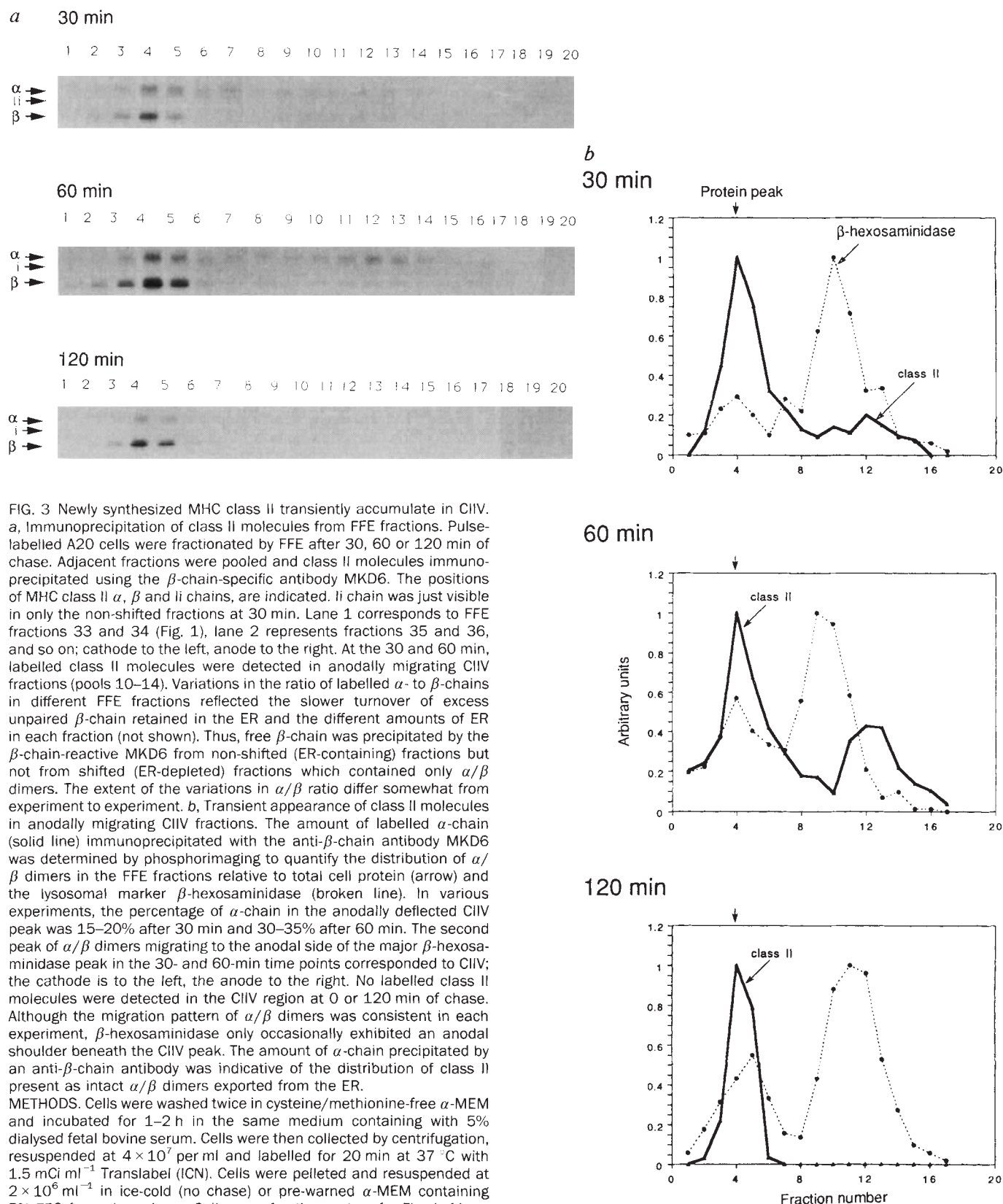


FIG. 3 Newly synthesized MHC class II transiently accumulate in CIIV. **a**, Immunoprecipitation of class II molecules from FFE fractions. Pulse-labelled A20 cells were fractionated by FFE after 30, 60 or 120 min of chase. Adjacent fractions were pooled and class II molecules immunoprecipitated using the β -chain-specific antibody MKD6. The positions of MHC class II α , β and li chains, are indicated. li chain was just visible in only the non-shifted fractions at 30 min. Lane 1 corresponds to FFE fractions 33 and 34 (Fig. 1), lane 2 represents fractions 35 and 36, and so on; cathode to the left, anode to the right. At the 30 and 60 min, labelled class II molecules were detected in anodally migrating CIIV fractions (pools 10–14). Variations in the ratio of labelled α - to β -chains in different FFE fractions reflected the slower turnover of excess unpaired β -chain retained in the ER and the different amounts of ER in each fraction (not shown). Thus, free β -chain was precipitated by the β -chain-reactive MKD6 from non-shifted (ER-containing) fractions but not from shifted (ER-depleted) fractions which contained only α/β dimers. The extent of the variations in α/β ratio differ somewhat from experiment to experiment. **b**, Transient appearance of class II molecules in anodally migrating CIIV fractions. The amount of labelled α -chain (solid line) immunoprecipitated with the anti- β -chain antibody MKD6 was determined by phosphorimaging to quantify the distribution of α/β dimers in the FFE fractions relative to total cell protein (arrow) and the lysosomal marker β -hexosaminidase (broken line). In various experiments, the percentage of α -chain in the anodally deflected CIIV peak was 15–20% after 30 min and 30–35% after 60 min. The second peak of α/β dimers migrating to the anodal side of the major β -hexosaminidase peak in the 30- and 60-min time points corresponded to CIIV; the cathode is to the left, the anode to the right. No labelled class II molecules were detected in the CIIV region at 0 or 120 min of chase. Although the migration pattern of α/β dimers was consistent in each experiment, β -hexosaminidase only occasionally exhibited an anodal shoulder beneath the CIIV peak. The amount of α -chain precipitated by an anti- β -chain antibody was indicative of the distribution of class II present as intact α/β dimers exported from the ER.

METHODS. Cells were washed twice in cysteine/methionine-free α -MEM and incubated for 1–2 h in the same medium containing with 5% dialysed fetal bovine serum. Cells were then collected by centrifugation, resuspended at 4×10^7 per ml and labelled for 20 min at 37 °C with 1.5 mCi ml^{-1} Translabel (ICN). Cells were pelleted and resuspended at 2×10^6 ml^{-1} in ice-cold (no chase) or pre-warmed α -MEM containing 5% FBS for various times. Cells were fractionated as for Fig. 1. Membrane pellets from each FFE fraction were dissolved in 1 ml lysis buffer (0.5% NP 40, 300 mM NaCl, 50 mM Tris, pH 7.4, plus 10 mg ml^{-1} mix of leupeptin, chymostatin, aprotinin and pepstatin) and then centrifuged. mIgG was first precipitated with rabbit anti-mouse IgG (Zymed) and fixed *Staphylococcus aureus* (Staph A; Zymed) overnight at 4 °C and incubated for 1 h with Staph A and protein A-Sepharose (Pharma-

cia). Class II molecules were immunoprecipitated with MKD6 (anti-I-A^d mAb³⁵)-coated protein A-Sepharose (1 h, 4 °C). The protein A-Sepharose was washed 3 $\times$ for 5 min in lysis buffer and boiled in non-reducing Laemmli SDS-PAGE sample buffer before electrophoresis in 12% gels.

of MHC class II molecules at the surface is delayed for 1–3 hours⁷. During this lag, newly synthesized class II molecules are thought to reach the endocytic pathway and bind antigenic peptides^{6–8}. We next determined whether CIIV might be a site of class II accumulation. A20 cells were pulse-labelled for 20 min and fractionated by FFE after chasing for different times; class II molecules were then immunoprecipitated with the β -chain-reactive antibody MKD6. No labelled class II was found in anodally shifted fractions directly after the pulse, but by 30 min newly synthesized class II started to appear in CIIV (Fig. 3a). Labelled α/β dimers in CIIV reached a maximum by 60 min but disappeared by 120 min. At 2 h, labelled class II heterodimers were found only in non-shifted fractions, reflecting α/β dimers that have reached the cell surface. Differences in the ratio of labelled α - and β -chains in different FFE fractions indicated the presence of unpaired β -chain retained in the ER and thus restricted to the unshifted fractions (Fig. 3 legend).

Given the variations in the ratio of α/β -chains, we only considered the transport kinetics of α/β dimers. We determined the amount of α -chain precipitated using the anti- β antibody; by definition, this measured only intact α/β dimers in each FFE fraction at each time point. As shown in Fig. 3b, labelled α -chain in CIIV fractions increased between 30 and 60 min, reaching a maximum of 30–35% of the total labelled class II. This was much greater than the 1–2% α -chain detected in CIIV fractions at equilibrium (Fig. 1). Thus, newly synthesized α/β dimers transiently accumulated in CIIV fractions. At 30 and 60 min, some labelled class II was also detected in intermediately deflected fractions containing Golgi and endosomes, confirming that class

II molecules were probably also passed through the Golgi and endosomes *en route* to the plasma membrane.

If newly synthesized α/β dimers were transported to the cell surface after leaving CIIV, then surface appearance would be complete after a 1–2-h delay. As this was more rapid than the ~3-h delay in human lymphoblasts⁷, we determined the rate of surface appearance of class II molecules in A20 cells. The kinetics of insertion were measured using cell-surface biotinylation²⁶ at various times of chase after a 20-min pulse of ³⁵S-methionine. As shown in Fig. 4a, labelled α/β dimers first began to appear only after 60 min of chase and reached a plateau within 120 min. In contrast, both membrane (γ m) and soluble (γ s) forms of IgG heavy chain become maximally accessible to surface biotinylation after just 20 min.

These data were quantified and the rate of α/β dimer transport to the surface compared to the rate of transit through CIIV. The kinetics indicated that newly synthesized class II molecules first appeared transiently in CIIV and then at the cell surface (Fig. 4b). Taking into account the inefficiency of the biotinylation assay (Fig. 4 legend)²⁶, the entire cohort of labelled class II molecules became accessible to surface biotinylation by 2 h. Thus, virtually all of the newly synthesized α/β dimers that reach CIIV—representing for at least 50–60% of the total (see below)—must subsequently appear on the plasma membrane rather than being degraded. Indeed, there was no degradation of labelled α - or β -chains over this time (Fig. 4a). Thus the delay in surface appearance of α/β dimers probably reflected the 0.5–1-h transient accumulation in CIIV. Newly synthesized membrane immunoglobulin, which was not delayed during

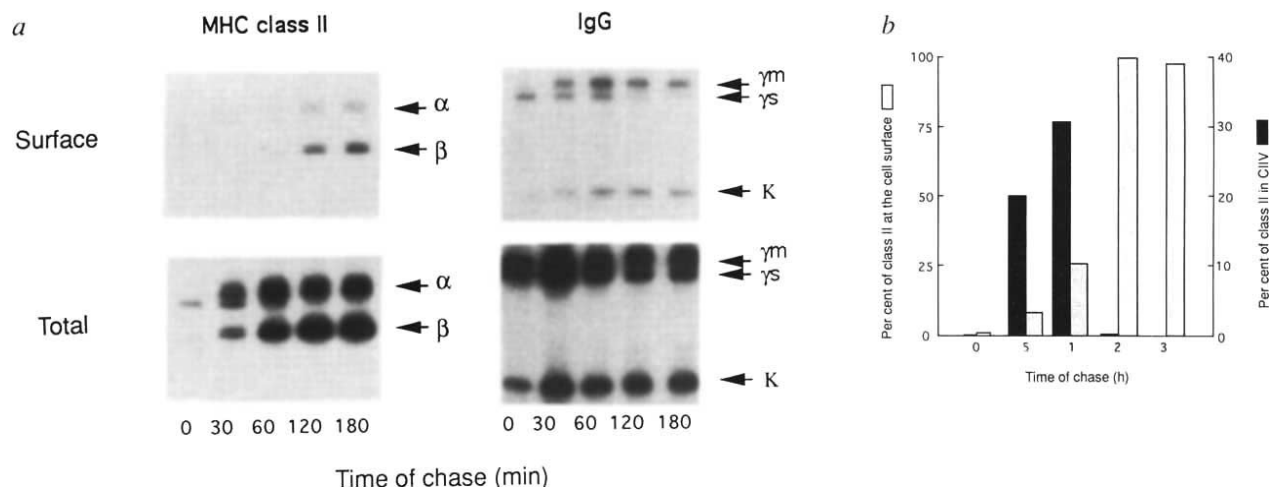


FIG. 4 Transport of newly synthesized MHC class II to the cell surface is delayed in murine B-lymphoma cells. **a**, Kinetics of appearance on the plasma membrane. A20 cells were pulsed with ³⁵S-methionine and chased at 37 °C. At the indicated times, cells were surface-biotinylated using *N*-hydroxysuccinamide-S-S-biotin (NHS-SS-biotin) to derivatize proteins reaching the plasma membrane. After lysis in detergent, mIgG and MHC class II molecules were sequentially immunoprecipitated ('total'). To determine the amount of each protein that had reached the surface, mIgG and class II molecules were then eluted from the immunoadsorbents with SDS and re-precipitated using streptavidin-agarose beads ('surface'). Relative to mIgG, the appearance of newly synthesized class II molecules on the cell surface was delayed by 2 h. A20 cells produce equal amounts of the membrane (γ m) and secreted (γ s) forms of IgG2a heavy chain and κ light chain; γ s became accessible to biotinylation before γ m but was rapidly released from the cells. β -chain was more susceptible to biotinylation than α -chain and thus appeared to be more heavily labelled on the 'surface' gels upon recovery of the free SDS-dissociated chains by streptavidin-agarose. **b**, Appearance of class II molecules in CIIV precedes appearance on the surface. The percentage of MHC class II at the plasma membrane (α - and β -chain) was corrected for the inefficiency of biotinylation using established procedures^{26,36–38} (judged at 15% of total, see Methods) and the kinetics of class II appearance was then compared to the appearance

of α -chain in CIIV at 0–2 h of chase. Newly synthesized class II molecules rapidly but transiently appeared in CIIV fractions. In contrast, after a lag, labelled class II molecules accumulated on the plasma membrane.

METHODS. Cell-surface biotinylation of pulse-labelled A20 cells was done at each chase time essentially as described²⁶ using cells suspended in 1 ml PBS and 2 mg ml⁻¹ NHS-SS-biotin (Pierce) for 2 min on ice. Class II molecules were precipitated with MKD6 (as for Fig. 2) and protein A-Sepharose beads boiled for 2 min in 100 μ l PBS containing 2% SDS; 25 μ l were transferred to SDS-PAGE sample buffer (total) and 75 μ l diluted into 1 ml PBS containing 2% NP-40, 0.1% SDS, 300 mM NaCl, 10 mM Tris, pH 8.6, then resuspended in 20 μ l sample buffer and boiled for 5 min. In control experiments, the efficiency of the biotinylation procedure at recovering class II molecules was directly determined using cells surface-labelled by lactoperoxidase-catalysed radioiodination. Under these conditions, 100% of the labelled α/β dimers were, by definition, accessible to biotinylation. Streptavidin-agarose reproducibly recovered ~15% of the ¹²⁵I- α/β dimers and ¹²⁵I-mIgG that were recovered by immunoprecipitation with MKD6. These values are typical of many membrane proteins^{26,35,36}.

transport from the *trans*-Golgi network, did not accumulate in CIIV before reaching the plasma membrane (not shown).

Removal of the Ii chain

We next asked whether newly synthesized α/β dimers were complexes with Ii chain upon delivery to CIIV. Although little if any Ii chain was detected when α/β dimers were precipitated using MKD6, this antibody inefficiently precipitates α/β /Ii complexes. A more sensitive approach was to use Ii chain-specific antibodies. Immunoprecipitation with an antibody against the Ii chain cytoplasmic domain (In-1) detected labelled Ii chain in a peak that comigrated with the unshifted (ER-containing) fractions as well as slightly shifted fractions containing Golgi and early endosome markers (Fig. 5a, b). Labelled α -chain was coprecipitated and labelled β -chain was less intense, consistent with the slower turnover and larger pool of β -chain in the ER, as already discussed.

Only a little labelled Ii-chain was detected by FFE in the anodally shifted CIIV fractions containing labelled α/β dimers immunoprecipitated with anti- β -chain antibodies (Fig. 5b). Results were similar at chase times of 30–120 min and with an antibody (P4H5) to a luminal epitope of Ii chain (not shown). Thus, Ii-chain cleavage and dissociation from α/β dimers occurred before or just after reaching CIIV. Accordingly, class II molecules in CIIV should be competent for binding antigenic peptides.

Peptide-loaded α/β dimers in CIIV

We investigated whether CIIV could be a site of peptide loading onto class II molecules using the criterion that they should contain α/β dimers stable to SDS detergent at room temperature (compact dimers)²⁷. We first measured the overall kinetics of compact dimer formation for I-A α/β class II molecules. As shown in Fig. 6a, SDS-stable compact dimers (bands corresponding to M_r 50–55K from samples not boiled before electrophoresis on polyacrylamide gels (SDS-PAGE)) were detectable as early as 30 min of chase and reached a plateau at 60 min. SDS-stable dimers therefore form before class II molecules appear at the plasma membrane, kinetics that correlate with the time spent by newly synthesized α/β dimers in CIIV.

To detect compact dimers in CIIV, we fractionated pulse-labelled cells after various times of chase. Unshifted (U) and anodally shifted CIIV fractions were pooled, and immunoprecipitated I-A α/β complexes treated at room temperature (not boiled, NB) or boiled (B) before SDS-PAGE (Fig. 6b). As expected, there was only a little labelled class II in

CIIV fractions at 30 min of chase, but after 60 min SDS-stable complexes were detected in both the CIIV and the unshifted fractions (5–8% of total α/β dimers in each fraction were SDS stable). Given that the size of the plasma membrane compartment was much larger than the CIIV compartment, and that class II molecules spend a comparatively long time on the plasma membrane, it was not surprising that compact dimers were already detected on the cell surface by this time. Compact dimers disappeared from the CIIV fractions by 120 min, cocomitant with the exit of all newly synthesized class II molecules (Fig. 3). Although compact dimers accumulated on the plasma membrane (unshifted fractions), they did not reappear later in CIIV fractions. Thus, once labelled α/β dimers have left CIIV and arrived at the cell surface, they do not return to CIIV by endocytosis. Similar results were obtained for class II I-E dimers, except that a greater fraction (15–20%) of I-E α/β complexes were SDS-stable in CIIV at 60 min (Fig. 6c).

Discussion

We have discovered a new population of vesicles, distinct from conventional endosomes and lysosomes, which act as intermediates in the intracellular transport of MHC class II molecules. Although their role in antigen processing remains to be directly determined, CIIV fulfill four criteria for being an intracellular site where MHC class II encounters and binds peptide antigen: (1) they receive antigen internalized by endocytosis; (2) they transiently accumulate newly synthesized MHC class II molecules before their arrival at the plasma membrane; (3) class II molecules in CIIV are no longer associated with Ii chain and are therefore available to bind antigenic peptides, and (4) at least some α/β dimers in CIIV are SDS-stable and are therefore loaded with peptide.

In A20 cells most newly synthesized class II molecules are probably transported through CIIV on their way to the plasma membrane. Assuming the half-time for transit through CIIV is ~ 0.5 h, because 30% of α/β dimers labelled during a 20 min pulse are found in CIIV after a 1-h chase, then at least 50–60% of the newly synthesized dimers should pass through CIIV. As <5% of pulse-labelled class II molecules in A20 cells are degraded during the first 2 h of chase, most dimers that enter

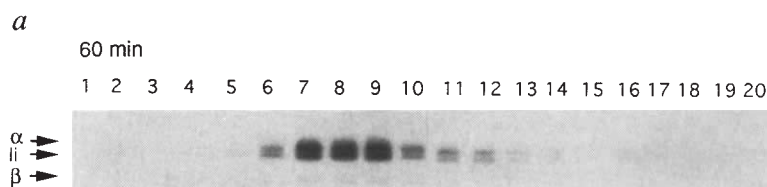
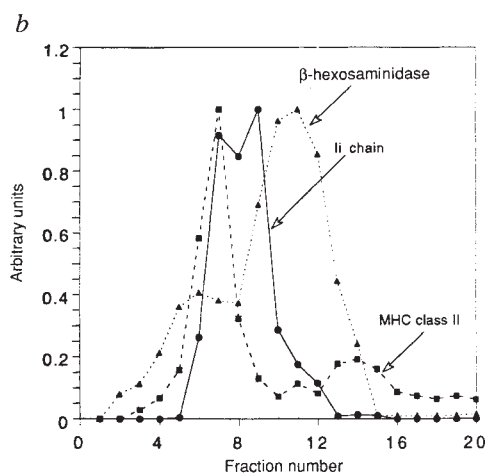


FIG. 5 Ii chain does not accumulate in CIIV. a, Immunoprecipitation of labelled Ii chain from FFE fractions. Metabolically labelled A20 cells were fractionated by FFE after 60 min of chase to maximize accumulation of newly synthesized class II molecules in CIIV (Fig. 3). Ii chain was immunoprecipitated from the FFE fractions using a monoclonal antibody (In-1) against the Ii chain-cytoplasmic domain. In this experiment, CIIV migrated slightly more to the anode than in Fig. 3 (see b). b, Distribution of labelled Ii chain. Bands in the gel shown in a were quantified by phosphor-imaging and the Ii chain distribution was plotted relative to total cell protein, β -hexosaminidase and MHC class II (determined by immunoprecipitation with MKD6). Ii chain was found in a single broad peak that overlapped but was more anodally-deflected than the major class II and protein peaks (which marked the positions of ER and plasma membrane). The anodally deflected region of the Ii peak overlapped the positions in which Golgi and some endosomal markers are found (Fig. 1). The Ii chain distribution was entirely distinct from the lysosomal (main β -hexosaminidase peak) and CIIV peaks; only small amounts of Ii chain were found in CIIV. Similar results were obtained after 30 and 120 min of chase, except that >80% of the total cell Ii chain had been degraded by 120 min. Results were identical when an antibody against a luminal Ii determinant (P4H5) was used, indicating that the FFE procedure did not selectively modify the Ii cytoplasmic domain. Cathode, left; anode, right.



CIIV must then go on to the cell surface. This may not be the case in other B cells in which most newly synthesized class II is rapidly degraded²³; such cells have large pools of class II molecules in heavy-density late endosomes or lysosomes (R. Germain, personal communication). Although α/β dimers in late endocytic compartments can be stabilized by peptide binding, they still fail to reach the plasma membrane as efficiently as α/β dimers in CIIV²³.

Whereabouts on the intracellular pathway of class II transport are CIIV likely to function? As α/β chains in CIIV are terminally glycosylated, they must pass through the Golgi complex. Another clue comes from the fact that α/β dimers in CIIV are not associated with Ii chain. Although Ii chain degradation and removal may occur in CIIV, Ii chain targets α/β dimers from the *trans*-Golgi network to endosomes, where it is dissociated by low pH and/or protease activity². Moreover, treatment of A20 cells with leupeptin, a protease inhibitor that blocks Ii chain cleavage, reversibly delays the appearance of newly synthesized class II molecules in CIIV and causes accumulation of α/β /Ii complexes in endosomes (our unpublished results). CIIV may therefore receive class II molecules after delivery to an endosome compartment.

The formation of CIIV may be analogous to the formation of post-endosomal compartments such as synaptic vesicles and transcytotic vesicles²⁸; CIIV would bud from the endosomal membrane and selectively include α/β dimers and possibly other membrane proteins, such as membrane IgG, that are involved in antigen processing. Receptors not directly involved in presentation, such as the transferrin receptor, might return from endosomes by means of constitutive recycling vesicles. At least some Tfn-R must also reach CIIV. As inclusion in recycling vesicles could depend on specific targeting signals²⁹, transport to CIIV may require an additional targeting determinant. Alternatively, it is possible that CIIV are not distinct from constitutive recycling vesicles and that class II molecules contain retention signals that slow their transit through this compartment. Ii chain has been proposed to contain such a signal³⁰. These two possibilities are not mutually exclusive.

Although CIIV are accessible to immune complexes internalized by means of Fc receptors, most ligand is delivered instead to endosomes and lysosomes and degraded completely²². Likewise, transferrin and its receptor are mainly restricted to early endosomes and recycling vesicles, although some Tfn-R was detected in CIIV. Similarly, in neuroendocrine cells and epithelial cells, only small fractions of internalized tracers reach post-endosomal synaptic vesicles and transcytotic vesicles, respectively³¹. Perhaps the internal volumes of synaptic vesicles and CIIV are smaller than the endosomes from which they are derived. In B cells, selective delivery of antigen to CIIV may occur via antigen receptors; indeed, our preliminary results indicate that both membrane IgG and IgM can efficiently enter CIIV. The fate of the majority of internalized molecules may not always follow the most useful pathway.

The relationship between CIIV and class II-positive vesicles in other B-cell lines such as MIIC in human lymphoblasts¹¹ is not yet known. These are morphologically similar, but it is unclear whether MIIC and CIIV represent a single compartment. MIIC have features in common with lysosomes and are not obviously intermediates in the transport of α/β dimers to the plasma membrane rather than to lysosomes. In contrast, CIIV appear to be intermediates in class II transport to the surface, contain markers of receptor recycling (Tfn-R) and, unlike MIIC, are devoid of the lysosomal membrane protein lgp-A (unpublished). Even if CIIV are found to play a role in peptide binding to class II molecules, they need not be the only intracellular site where this can occur.

Targeting of newly synthesized class II molecules to CIIV after delivery to endosomes would enable α/β dimers to encounter internalized antigens and then to delay surface transport to facilitate peptide binding. Such a mechanism would explain why newly synthesized class II molecules are accessible to neuroaminidase bound to recycling receptors^{7,32} but, unlike recycling receptors, show a lag in transport to the plasma membrane. It is also consistent with known functions of endosomes in other cell types. By sorting the protagonists in antigen presentation out of the constitutive endocytic pathway and delaying their transport to the surface, professional APCs may improve their ability to associate antigenic peptides with MHC class II molecules. □

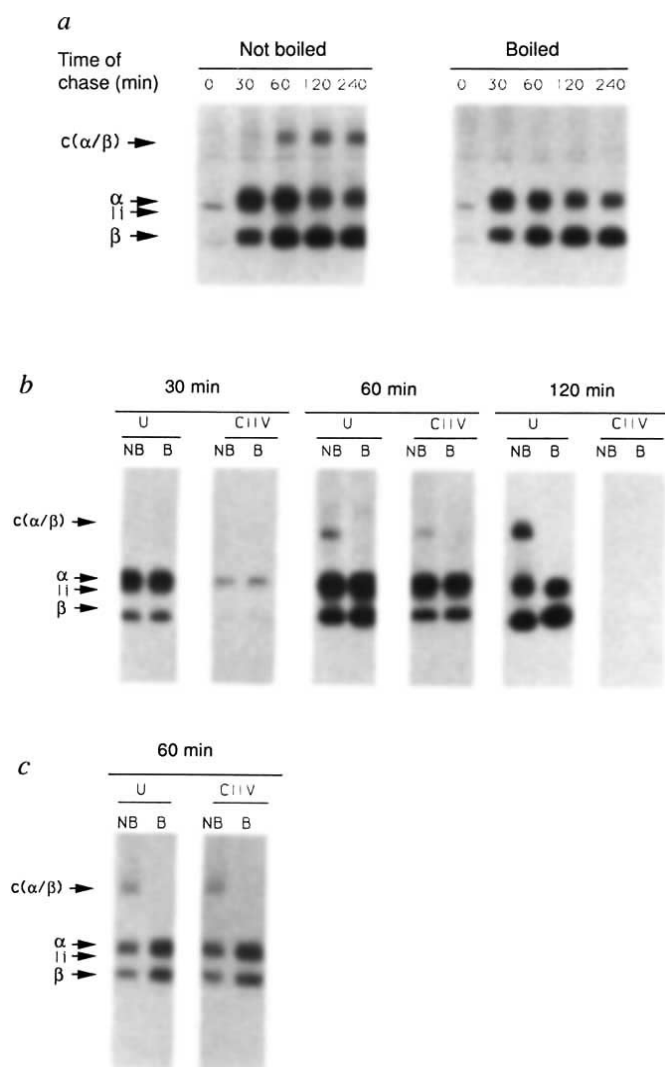


FIG. 6 MHC class II-peptide complexes transiently accumulate in CIIV. **a**, Formation of SDS-stable α/β dimers. A20 cells were pulse-labelled for 20 min, chased for the indicated time and MHC class II (I-A) molecules immunoprecipitated. Labelled class II molecules were released from the immunoabsorbent by incubation in 3% SDS (Laemmli sample buffer) for 60 min at room temperature. Samples were boiled for 3 min (B) or not boiled (NB) before SDS-PAGE. The electrophoretic mobility of SDS-stable compact dimers ($c[\alpha/\beta]$) was slower than free α - or β -chains²³. The fraction of SDS stable class II in this experiment was 16%, and reached a maximum after 60 min. **b** and **c**, SDS-stable α/β dimers are transiently detected in CIIV. Pulse-labelled cells were chased for 30, 60 or 120 min and fractionated by FFE. Pooled fractions corresponding to the unshifted plasma membrane/ER peak (U) or to the anodally shifted CIIV peak were lysed and class II I-A (**b**) or I-E (**c**) molecules immunoprecipitated. Samples were boiled (B) or not boiled (NB) before SDS-PAGE. The positions of SDS-stable compact α/β dimers ($c[\alpha/\beta]$), Ii chain (not visible), and free MHC class II α - and β -chains are indicated. At the 60 min, SDS-stable I-A and I-E were observed and accounted for ~8 and ~25% of the total labelled class II molecules, respectively.

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Isolation and characterization of the intracellular MHC class II compartment

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An intracellular compartment has been isolated to which MHC class II molecules are transported on their way to the plasma membrane. They arrive with an associated invariant chain which is then proteolytically processed while MHC class II molecules acquire antigenic peptide. These loaded class II molecules then leave the compartment devoid of invariant chain and bound for the plasma membrane. This compartment represents a new stage in the endocytic/lysosomal pathway.

MAJOR histocompatibility complex (MHC) class II molecules present antigenic peptides to CD4-positive T cells¹. The peptides bound to MHC class II are derived mainly from proteins that have entered the endocytic pathway, whereas MHC class I molecules present peptides derived from proteins synthesized in the cytoplasm^{2,3}.

At the plasma membrane, MHC class II molecules consist of two non-identical glycoproteins, the α - and β -chains⁴. Early in biosynthesis, class II molecules associate with the invariant chain (Ii)⁵, which is thought to perform three functions: (1) it guides folding of class II molecules to facilitate their exit from the endoplasmic reticulum (ER)⁶; (2) it prevents premature binding of antigenic peptides to class II molecules^{7,8}; and (3) it functions as a targeting signal for delivery of class II molecules to endocytic compartments^{9,10}. Following deposition in the endocytic pathway, the class II-associated Ii is proteolytically processed^{11,12}.

The endocytic compartment exists as a complex network of tubulo-vesicular structures^{13,14}, the precise structure and composition of which is still unknown. The endosomal/lysosomal system is thought to consist of several subcompartments, including early endosomes, late endosomes, and dense lysosomes^{15–18}. In addition, multivesicular bodies are found in a number of cell types and may mediate traffic of endocytosed material between early and late endosomes¹⁷.

Using immunocytochemical methods, MHC class II molecules are found in post-Golgi, endosomal and lysosomal-related compartments^{12,19,20}. To characterize the intracellular site within the endosomal/lysosomal system where MHC class II molecules and Ii reside, we have analysed their location in the human melanoma cell line Mel JuSo by subcellular fractionation and density-gradient electrophoresis (DGE). Mel JuSo cells and related melanoma and melanocyte lines that are class II-positive²¹ can induce a strong MHC class II-dependent T-cell response (A. Bakker, G. Adema and J.P., unpublished results). We report here that in Mel JuSo cells complexes of MHC class II molecules and Ii fragments are present in an intracellular

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compartment distinct from early and late endosomes and different from dense lysosomes. Here MHC class II dimers acquire their stability to exposure to SDS at ambient temperature, a feature associated with the peptide-liganded state of class II molecules^{22,33}. From this compartment, which may be identical to the morphologically characterized MHC class II compartment²⁰, MHC class II molecules exit to the plasma membrane.

Density gradient electrophoresis

Endosomes are identified by cytochemistry and biochemically after labelling with endocytic tracers¹⁷. The surface charge of lysosomal and endosomal compartments is more negative than that of most other subcellular organelles¹⁸, so free-flow electrophoresis can be used to isolate lysosomal and endosomal compartments^{24,25}.

A density gradient was combined with electrophoresis to separate endosomal/lysosomal compartments from crude microsomes in a single step. Early and late endosomes of Mel JuSo cells were labelled with horseradish peroxidase (HRP)^{12,17}, membranes were prepared (Fig. 1 legend), subjected to DGE, and the distribution of markers for the various subcellular compartments determined. Two endosomal populations, consisting of early and late endosomes, were resolved (Fig. 1a).

Lysosomal compartments were identified by β -hexosaminidase activity and migrated towards the anode, well separated from both early and late endosomes. To analyse the migration of plasma membrane components, Mel JuSo cells were surface-labelled on ice with ¹²⁵I. The plasma membrane was well resolved from endosomal and lysosomal organelles by DGE (Fig. 1b). The position of the ER was established by pulse-labelling Mel JuSo cells for 2 min with ³⁵S-methionine/cysteine before homogenization and DGE. The ER migrated at a position distinct from that of endosomes and lysosomes but overlapped partially with the plasma membrane (Fig. 1c). Golgi membranes, identi-

fied by enriched galactosyltransferase activity, were slightly shifted anodically and overlapped with early endosomal membranes, in agreement with other subcellular electrophoretic separations²⁴. The intracellular distribution of MHC class II molecules, see below, is shown in Fig. 1 (shaded).

Early and late endosomes can thus be separated from a crude microsomal preparation in a single step by density gradient electrophoresis. Lysosomes overlap but do not comigrate with the late endosomal fraction.

Detection of MHC class II compartments

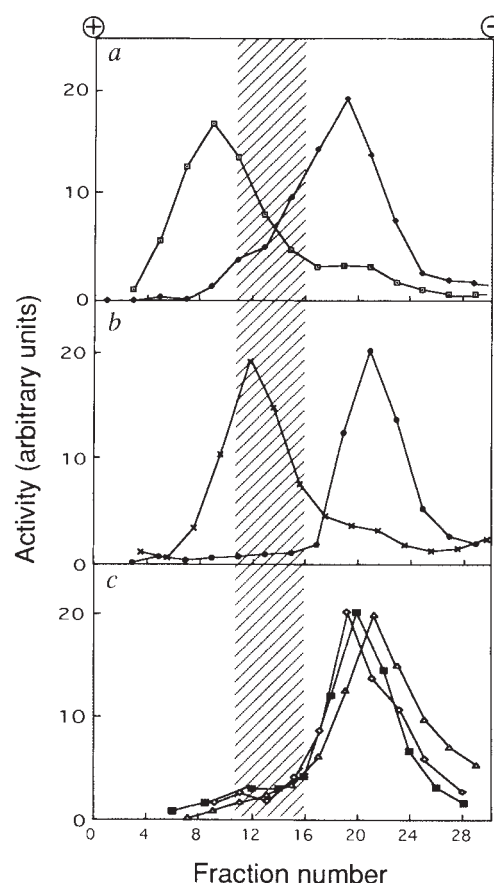
To analyse the subcellular location of MHC class II molecules, Mel JuSo cells incubated with HRP were homogenized and the microsomal fraction subjected to DGE. The distribution of MHC class II molecules at steady state was determined by immunoblotting (Fig. 2a). MHC class II molecules were present in two distinct populations; the first comigrated with the plasma membrane (Fig. 1b), and the second migrated between early and late endosomes. A small fraction of intracellular class II molecules overlapped with both early and late endosomes (Fig. 1a).

Can the intracellular compartments containing MHC class II molecules (as defined by their migration in fractions 11–16 after DGE) be reached from the outside by endocytosis? Mel JuSo cells were allowed to internalize HRP for 4 min and then chased for the times indicated (Fig. 2b). After a 10-min chase, HRP cofractionates with the intracellular MHC class II molecules (Fig. 2b). After further purification by sucrose-gradient sedimentation of the DGE-defined intracellular MHC class II compartments, the bulk of the internalized marker again cofractionates with the MHC class II-positive fractions (Fig. 5a).

Marker proteins for the endosomal/lysosomal pathway include the lysosomal-associated membrane protein LAMP³⁶ and the cation-independent mannose 6-phosphate receptor (CI-MPR)¹⁵. The location of both marker proteins was analysed after DGE. Most of the LAMP immunoreactivity was found at

FIG. 1 Subcellular fractionation of Mel JuSo organelles by density-gradient electrophoresis (DGE). Mel JuSo cells were allowed to internalize horseradish peroxidase (HRP) at 37 °C for 4 min to label early endosomes, or for 4 min followed by a 30-min chase to label late endosomes in normal medium. Microsomes prepared from Mel JuSo cells were electrophoresed for 35 min at 10 mA and 0.3-ml fractions collected. a, Detection of early (filled diamonds) and late (open squares) endosomes by HRP activity as described¹⁷. b, Detection of lysosomes by β -hexosaminidase activity³⁹ (cross) and of plasma membrane (filled circles) by ¹²⁵I label. c, Golgi membranes assayed by galactosyl transferase activity⁴⁰ (diamonds), endoplasmic reticulum, as assayed by the presence of ³⁵S after pulsing the cells for 2 min with ³⁵S-methionine/cysteine (filled squares), and total protein determined according to ref. 41 (triangles). The shaded region represents fractions containing the intracellular MHC class II molecules (Fig. 2).

METHODS. Monolayers of Mel JuSo cells were scraped in homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose, pH 7.4) and collected by centrifugation at 136g for 7 min. The cell pellet was resuspended in 1 ml homogenization buffer and homogenized at 4 °C by passing 20 times through a 22G1 1/4 needle fitted on a 1-ml plastic syringe¹². After centrifugation for 15 min at 840g at 4 °C, the postnuclear supernatant (PNS) was treated with trypsin for 5 min at 37 °C at a concentration of 25 μ g per mg protein¹⁶. Digestion was stopped by cooling on ice and addition of 100 μ g soybean trypsin inhibitor per mg. The PNS was subsequently centrifuged at 100,000g for 60 min at 4 °C and resuspended in 0.5 ml 5% Ficoll-70 in homogenization buffer. The electrophoretic device consisted of a Perspex cylinder separated from the bottom buffer reservoir by a cationic-permeable membrane at the bottom of the tube⁴³. The sample was layered in the middle of a linear gradient from 10 to 0% Ficoll-70 in electrophoresis buffer. Plasma membrane labelling was achieved by radioiodination of cells on ice by the lactoperoxidase–glucose oxidase method⁴⁴.



the position of the intracellularly located MHC class II molecules (Fig. 2c). The small amount of LAMP immunoreactivity detected in fractions 22–24 may represent ER-resident and/or LAMP molecules present at the plasma membrane²⁶.

The bulk of the CI-MPR was present in fractions containing early endosomes/Golgi compartments (Fig. 2d), probably reflecting the extensive recycling of the CI-MPR through the *trans*-Golgi network²⁷. But fractions containing MHC class II-positive intracellular compartments (fractions 11–16) also contain considerable amounts of the CI-MPR.

We conclude that the MHC class II molecules are present in an intracellular compartment that is distinct from early and late endosomes. Internalized proteins have access to these compartments and comigrate partially with the CI-MPR, the lysosomal marker β -hexosaminidase (see later), and the lysosomal-associated protein LAMP.

Transport of MHC class II/Ii complexes

To investigate the nature of the intracellular target compartment of newly synthesized class II molecules and Ii, we either pulsed Mel JuSo cells with ³⁵S-methionine/cysteine for 10 min or labelled them for 20 min and chased for the times indicated in Fig. 3. After DGE of the homogenates, MHC class II molecules were immunoprecipitated from the different fractions. For longer chase times, transferrin receptor and MHC class I molecules can be used as markers for early endosomes and plasma membrane, respectively. As expected, after a short pulse most of the class II molecules, as well as MHC class I and transferrin receptor molecules, migrated with the ER marker (Fig. 3a). A proteolytic fragment of the class II-associated Ii (P25) that was generated during or directly after biosynthesis¹², and P41, an

alternatively spliced form of Ii (ref. 28), were both present in the class II immune complexes. With increasing chase times, the class II/Ii positive compartments migrated further towards the anode (Fig. 3). After 2 and 4 hours of chase, MHC class II molecules associated with the Ii fragments P22, P18 and P12 migrated between early and late endosomes, at the same position as the cohort of intracellularly located class II molecules determined by immunoblotting (Fig. 2). After 2 and 4 h chase, biosynthetically labelled transferrin receptors and MHC class I molecules migrated at the positions of early endosomal compartments and plasma membrane, respectively.

MHC class II molecules have a long half-life (15–50 h)²⁹. In Mel JuSo cells, class II molecules remain associated with N-terminal Ii fragments within the endocytic pathway¹². Later, these Ii fragments are further degraded and dissociated from class II molecules. The N-terminal fragments of Ii contain the sorting and retention signals for endosomal compartments^{9,10,30}, and removal of these Ii fragments by proteolysis can lead to the appearance at the plasma membrane of MHC class II molecules^{12,23}. After 48 h chase, biosynthetically labelled MHC class II complexes devoid of Ii fragments migrated predominantly at the position of the plasma membrane (Fig. 3e). The small intracellular fraction may represent MHC class II molecules internalized from the plasma membrane³¹. MHC class I molecules were no longer detected after 48 h chase; the position of the transferrin receptor was shifted slightly towards the anode compared to the 2- or 4-h chase points, and may be due to the transition of small amounts of transferrin receptor from early endosomes to later stages of the endocytic pathway³². Thus, newly synthesized class II/Ii complexes are transported to an intracellular compartment that is distinct from early and late

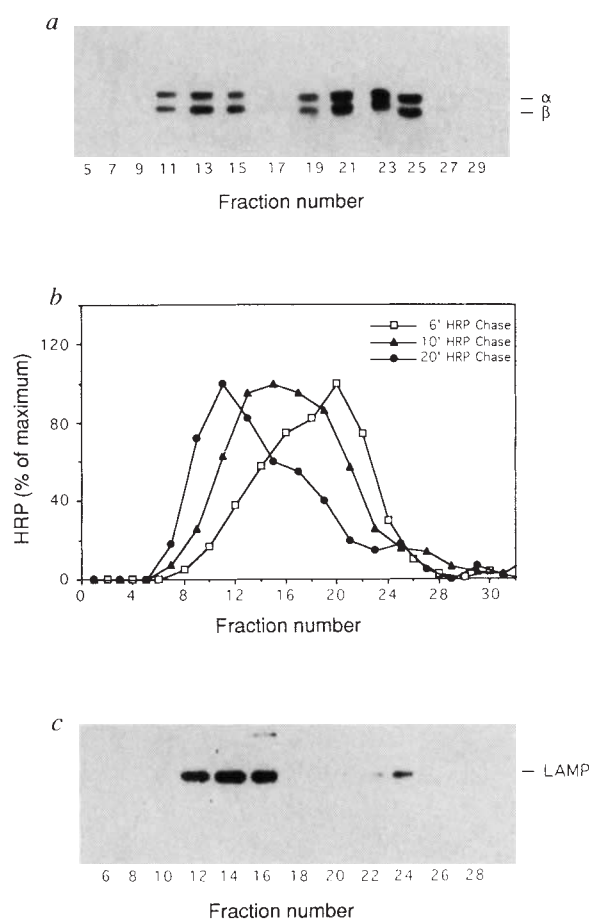


FIG. 2 a, Steady-state distribution of MHC class II molecules in subcellular organelles of Mel JuSo. Mel JuSo cells were homogenized, and after DGE of the postnuclear supernatant, proteins from the indicated fractions were precipitated with trichloroacetic acid (TCA), subjected to 12% SDS-PAGE and transferred to nitrocellulose. The presence of MHC class II molecules was detected using anti-class II antibodies^{45,46}. Quantification by densitometry revealed that $24 \pm 6\%$ (mean value from 3 independent experiments) was present in the intracellularly located membrane fractions 11–17. b, Distribution of HRP after different chase times. Mel JuSo cells were allowed to internalize HRP for 4 min, then chased in HRP-free medium for 6 (open squares), 10 (filled triangles) and 20 min (filled circles). Subcellular fractionation by DGE is described in Fig. 1 legend. c, Distribution of lysosome-associated membrane protein (LAMP) in subcellular organelles of Mel JuSo cells. Proteins from subcellular fractions after DGE were subjected to SDS-PAGE and transferred to nitrocellulose; LAMP was detected using anti-LAMP antibodies. d, Distribution of the cation-independent mannose 6-phosphate receptor (CI-MPR) in subcellular organelles of Mel JuSo cells. Cells were metabolically labelled for 20 min, followed by a 2-h chase. After homogenization and fractionation by DGE, the amount of CI-MPR in the fractions indicated was determined by immunoprecipitation using anti-CI-MPR antibodies followed by SDS-PAGE (6%) and fluorography and direct quantification from the gel by phosphor image analysis.

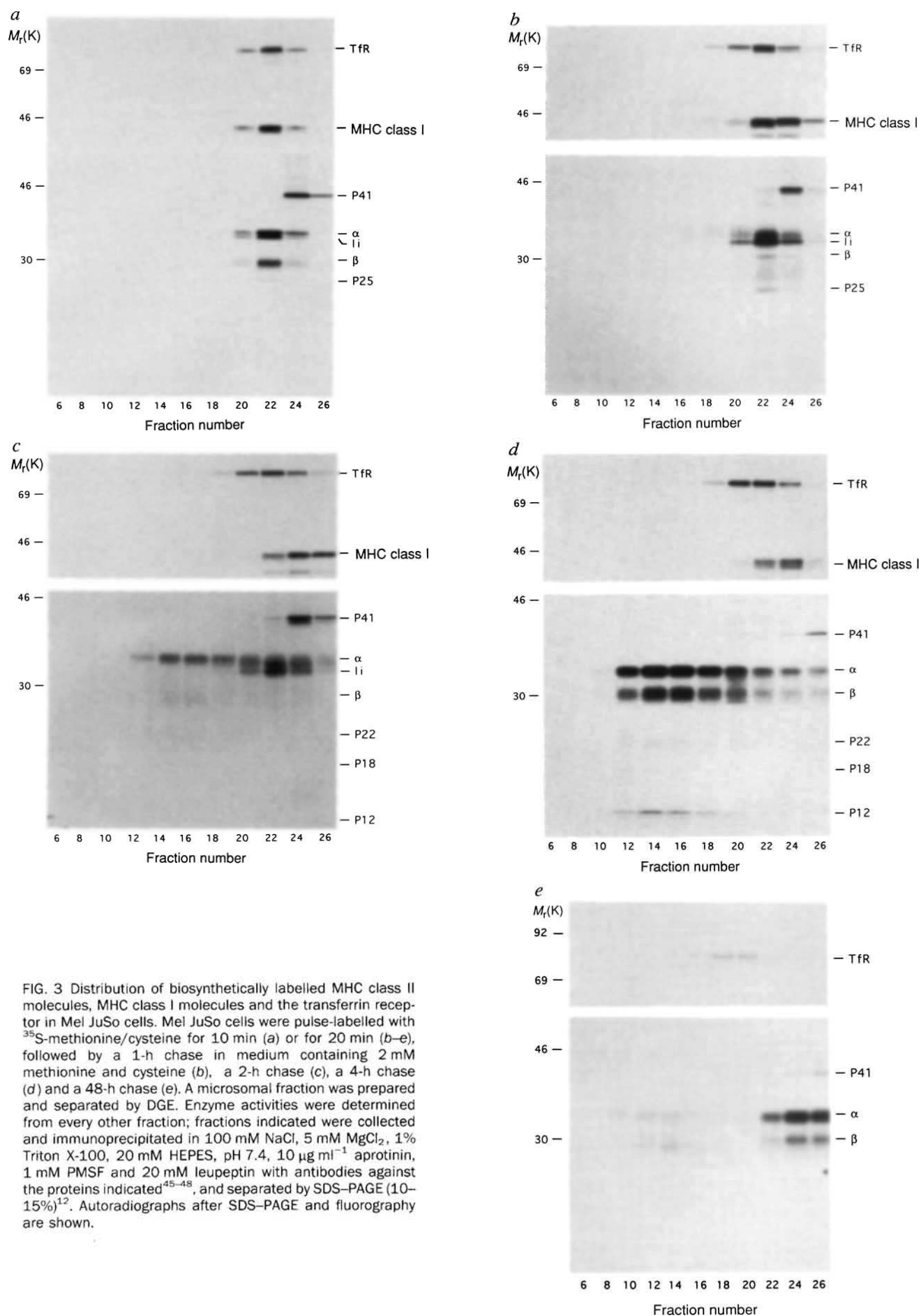


FIG. 3 Distribution of biosynthetically labelled MHC class II molecules, MHC class I molecules and the transferrin receptor in Mel JuSo cells. Mel JuSo cells were pulse-labelled with ^{35}S -methionine/cysteine for 10 min (a) or for 20 min (b–e), followed by a 1-h chase in medium containing 2 mM methionine and cysteine (b), a 2-h chase (c), a 4-h chase (d) and a 48-h chase (e). A microsomal fraction was prepared and separated by DGE. Enzyme activities were determined from every other fraction; fractions indicated were collected and immunoprecipitated in 100 mM NaCl, 5 mM MgCl_2 , 1% Triton X-100, 20 mM HEPES, pH 7.4, $10\text{ }\mu\text{g ml}^{-1}$ aprotinin, 1 mM PMSF and 20 mM leupeptin with antibodies against the proteins indicated^{45–48}, and separated by SDS-PAGE (10–15%)¹². Autoradiographs after SDS-PAGE and fluorography are shown.

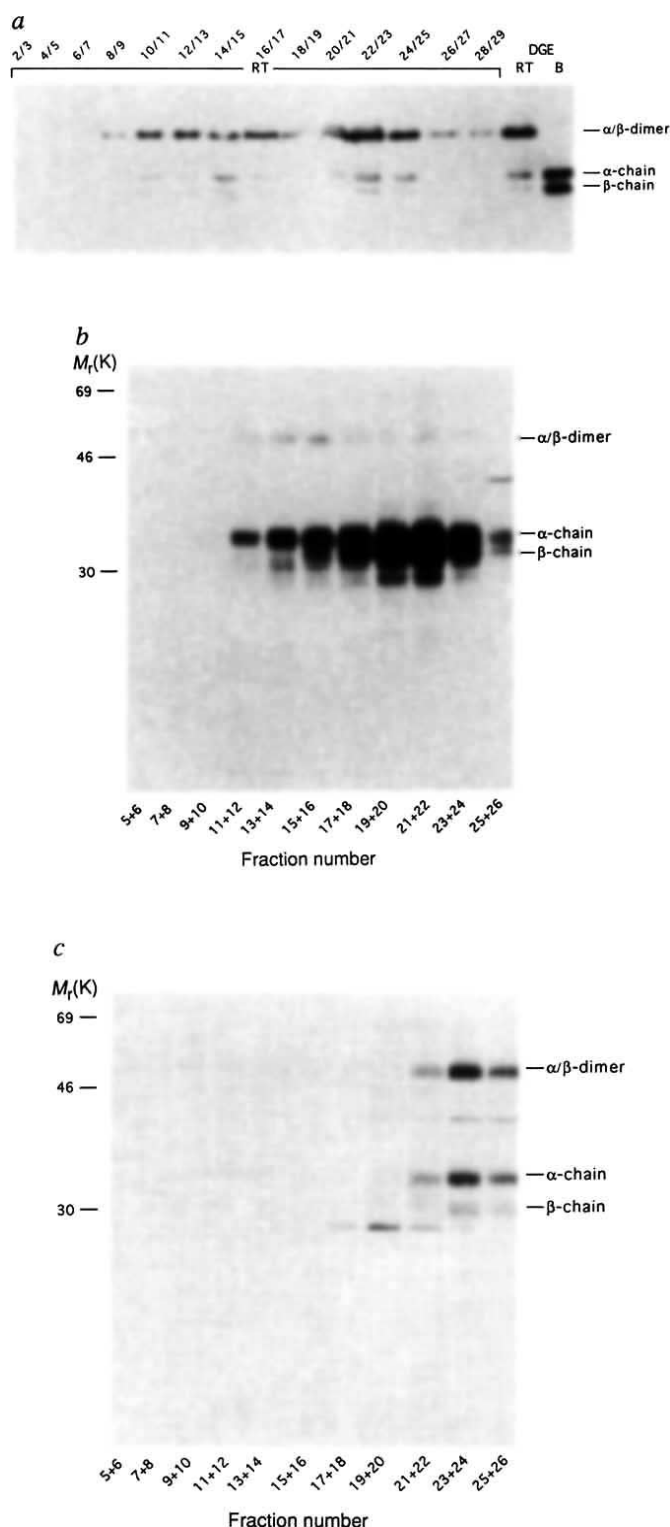


FIG. 4 **a**, Distribution of SDS stable α/β dimers in DGE fractions. Microsomes from Mel JuSo cells were separated by DGE as for Fig. 2. Before SDS-PAGE and immunoblotting, samples were allowed to stand for 30 min at room temperature (RT), or denatured at 95 °C (B, boiled). DGE, membrane fraction before density gradient electrophoresis. **b** and **c**, Presence of newly formed SDS-stable α/β dimers in the DGE-defined MHC class II-positive intracellular compartment. Mel JuSo cells were pulse-labelled with ^{35}S -methionine/cysteine for 20 min followed by a 2-h (**b**) or 48-h (**c**) chase. After homogenization and subcellular fractionation by DGE, proteins from the fractions indicated were immunoprecipitated with anti-class II antibodies and separated by SDS-PAGE after denaturation at ambient temperature. Shown are autoradiographs after SDS-PAGE and fluorography.

endosomes. In this compartment II is proteolytically processed from its carboxy terminus, after which class II molecules are transported to the plasma membrane.

Detection of SDS-stable α/β dimers

Peptide loading confers increased resistance of class II molecules to denaturation by SDS detergent^{22,33}, although not all peptide-occupied class II molecules are resistant to SDS³⁴. We analysed MHC class II molecules in DGE fractions by immunoblotting, following their resolution using polyacrylamide gel electrophoresis (SDS-PAGE) under mildly denaturing conditions (Fig. 4a). As expected³³, SDS-stable dimers were present in the fractions containing plasma membrane. The intracellular compartment enriched for class II molecules contained a similar proportion of SDS-stable dimers relative to monomers. Acquisition of SDS stability coincides with the deposition of class II molecules in post-Golgi compartments^{22,23}. In Mel JuSo cells, as in other cells, newly synthesized class II molecules acquire resistance to SDS ~2 h after biosynthesis (data not shown). To find out in which subcellular compartment this conversion takes place, cells that had been pulse-labelled and chased for 2 h were fractionated by DGE and the presence of SDS-stable MHC class II dimers was tested by immunoprecipitation followed by SDS-PAGE under mildly denaturing conditions. SDS-stable class II dimers were predominantly present in fractions 11–16 (Fig. 4b).

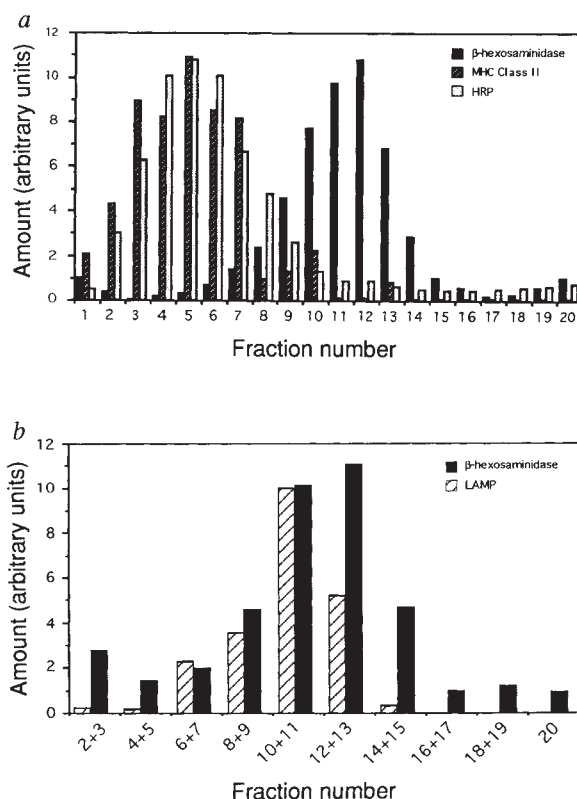


FIG. 5 Separation of MHC class II-positive compartments from lysosomes. Mel JuSo cells were allowed to internalize HRP for 4 min, followed by a 10-min chase. After homogenization and DGE, fractions containing MHC class II-positive intracellular compartments resolved by DGE (fractions 11–16; Fig. 2) were pooled and layered onto a linear sucrose gradient (0.6–1.6 M sucrose in DGE buffer). After centrifugation (5 h at 27,000 r.p.m. in a Beckman SW-41 rotor), 0.5-ml fractions were collected from the top; β -hexosaminidase (**a**, **b**) and HRP (**a**) activity was determined in each fraction. Proteins from each fraction were precipitated in 10% TCA/acetone, and separated by SDS-PAGE. Following transfer to nitrocellulose, class II molecules (**a**) and LAMP (**b**) were quantified in each fraction by immunolabelling and densitometry.

A small percentage of SDS stable dimers were present in fractions 19–22, probably representing the few class II dimers that either recycle through the Golgi or have reached the plasma membrane. After 48 h of chase, SDS-stable $\alpha\beta$ dimers appeared in plasma membrane fractions, as expected (Fig. 4c). We conclude that conversion of class II molecules to SDS-stable heterodimers is largely completed in this DGE-defined compartment; some unstable class II molecules may be degraded intracellularly rather than being transported to the plasma membrane³⁵.

Separation from lysosomes

The intracellular compartment containing MHC class II molecules partially overlaps with lysosomes in DGE (Figs 1 and 2).

To resolve further the class II-containing compartments, MHC class II-positive membrane fractions from Mel JuSo cells (fractions 11–16; Fig. 2a), were subjected to sucrose density gradient centrifugation (0.6–1.6 M sucrose). The distributions of β -hexosaminidase (representing lysosomes) and class II molecules were determined (Fig. 5a). MHC class II-containing fractions (light fractions, 1–7) were well resolved from the denser fractions containing β -hexosaminidase activity (fractions 9–14). The lysosome-associated membrane protein LAMP is present mainly in fractions containing dense lysosomes but also in the lighter MHC class II-positive fractions (Fig. 5b). We conclude that the MHC class II-containing intracellular compartment is different from lysosomes, although it is still positive for LAMP.

Proteins in the MHC class II compartment

The physical properties of the MHC class II-containing intracellular compartment suggest that it represents a compartment distinct from endosomes and lysosomes. Proteins in the different subcellular fractions were analysed by two-dimensional isoelectric focusing (IEF)/SDS-PAGE of proteins from total membranes (Fig. 6a) and from pooled fractions containing intracellular MHC class II molecules after DGE (Fig. 6b). After further purification on a sucrose gradient (Fig. 5), the protein profile shown in Fig. 6c is obtained. Four spots of roughly

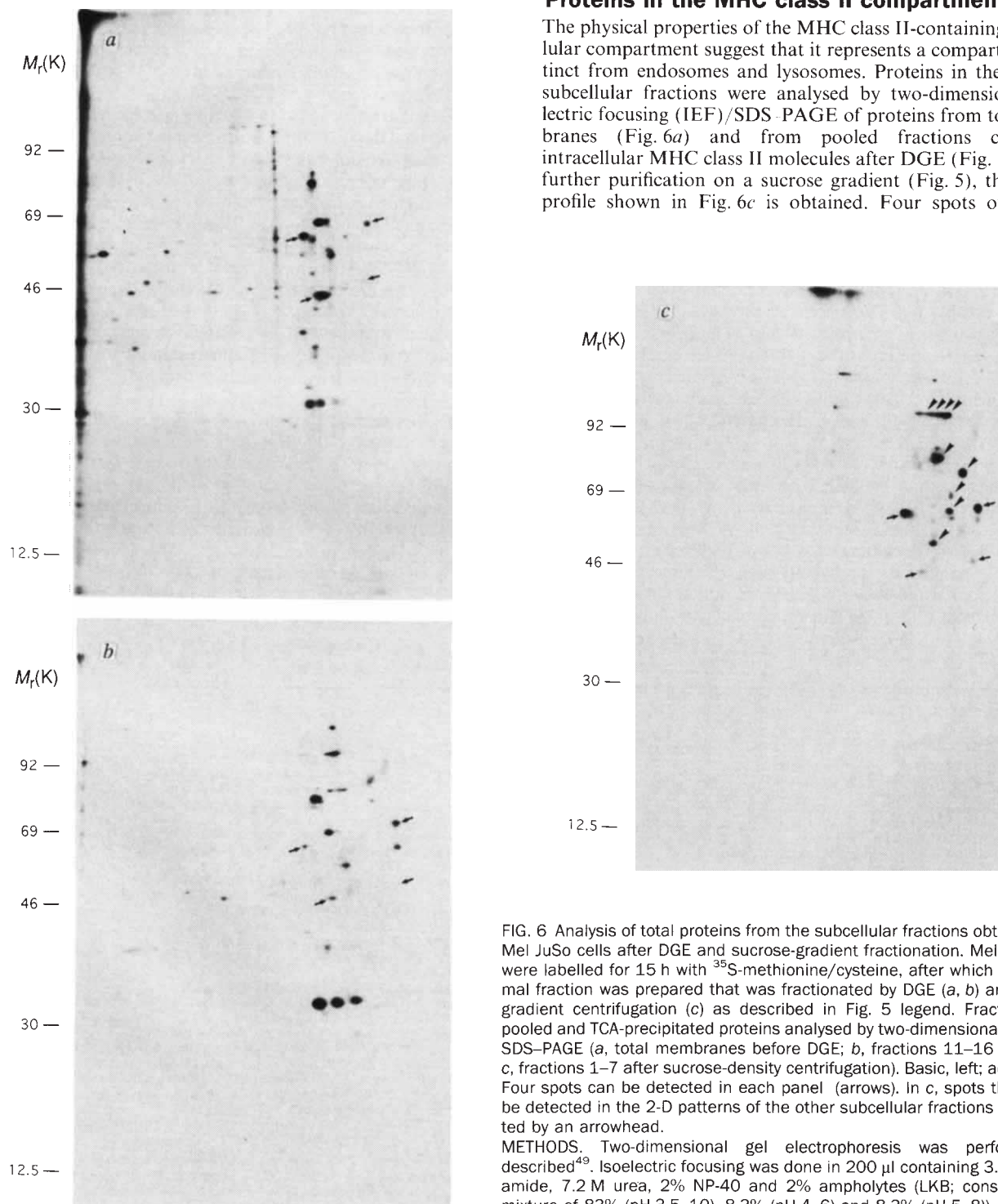


FIG. 6 Analysis of total proteins from the subcellular fractions obtained from Mel JuSo cells after DGE and sucrose-gradient fractionation. Mel JuSo cells were labelled for 15 h with ³⁵S-methionine/cysteine, after which a microsomal fraction was prepared that was fractionated by DGE (a, b) and sucrose gradient centrifugation (c) as described in Fig. 5 legend. Fractions were pooled and TCA-precipitated proteins analysed by two-dimensional (2-D) IEF/SDS-PAGE (a, total membranes before DGE; b, fractions 11–16 after DGE; c, fractions 1–7 after sucrose-density centrifugation). Basic, left; acidic, right. Four spots can be detected in each panel (arrows). In c, spots that cannot be detected in the 2-D patterns of the other subcellular fractions are indicated by an arrowhead.

METHODS. Two-dimensional gel electrophoresis was performed as described⁴⁹. Isoelectric focusing was done in 200 μ l containing 3.75% acrylamide, 7.2 M urea, 2% NP-40 and 2% ampholytes (LKB; consisting of a mixture of 83% (pH 3.5–10), 8.3% (pH 4–6) and 8.3% (pH 5–8)).

equivalent intensities are present in the $M_r=40\text{--}60\text{K}$ region (Fig. 6a–c, arrows). The pattern is strikingly different in the lighter fractions (Fig. 6c), which contain polypeptides not evident in Fig. 6a, or b. Thus, MHC class II molecules are transported to a compartment which houses proteins of a characteristic composition profile distinct from that of other subcellular fractions.

Discussion

We have identified an intracellular compartment in the human melanoma cell line Mel JuSo that contains MHC class II molecules and which may be similar to the morphologically defined MIIC²⁰.

Two populations of MHC class II-containing membrane fractions are resolved by DGE, one in the plasma-membrane-containing fractions and the other in fractions comprising an intracellular compartment comigrating with the lysosomal-associated membrane protein LAMP and containing protease activity, as deduced from the degradation of the class II-associated Ii. This intracellular pool of class II molecules only partially overlaps with early and late endosomes, is accessible to a fluid-phase marker and is separable from dense lysosomes. Two-dimensional IEF/SDS-PAGE analysis indicates that the MHC class II-containing compartment has a protein composition different from that of the other subcellular organelle fractions.

Newly synthesized class II molecules are found in this compartment 2–4 h after biosynthesis and are associated with N-terminal cytoplasmic fragments of the invariant chain; after longer chase times the majority of class II molecules, are present at the plasma membrane without Ii. The N-terminal cytoplasmic tail of Ii is known to contain sorting signals for endosomal compartments^{9,10,30}. Our findings are consistent with a function of Ii in targeting/retaining the MHC class II molecules intracellularly.

In this intracellular MHC class II-positive compartment, class II molecules acquire peptide, with only a small proportion of newly synthesized class II molecules being occupied at first, increasing until at steady state the bulk of them are loaded with peptide. This suggests that class II molecules are retained and that peptide acquisition is relatively slow. Unstable, non-peptide-occupied class II molecules might be degraded intracellularly³⁵.

In Mel JuSo cells there are only small amounts of class II molecules present in lysosomes¹²; class II molecules and Ii are

found in endosomes and in multivesicular bodies¹². These multivesicular bodies were identified by their characteristic morphology, because only a subset of them contained endosomal markers, including the CI-MPR. Our results are in good agreement with these earlier observations; although some class II/Ii complexes comigrated with markers for early and late endosomes, the major portion was present in the DGE-defined intermediate membrane fractions, which may be identical to multivesicular bodies.

This class II-positive intracellular compartment also resembles MIIC, another multivesicular-like structure²⁰. These MIIC are not lysosomes, they contain LAMP and can be reached by endocytic tracers. In the cell line JY, endocytic tracers reached the MIIC within 60 min, whereas in Mel JuSo cells the multivesicular bodies were loaded with tracers within 15 min, which agrees with our results and has been seen in other cells^{12,36}. The kinetics of internalization, which could vary between cell types, may differ from the transport kinetics of molecules to MIIC from the biosynthetic pathway.

This compartment may be a sorting site for MHC class II/Ii complexes, analogous to the multivesicular bodies for other cell-surface receptor molecules¹⁴. Ii, by virtue of its endosomal sorting/retention signals, may deliver and retain class II molecules in this compartment. After proteolysis of the Ii luminal domain, antigenic peptides bind to class II molecules (Fig. 4) for presentation at the plasma membrane.

The MHC class II compartment characterized here may be a specialized endosome, present in class II-positive antigen-presenting cells. The unique as-yet unidentified polypeptides that are enriched in the MHC class II-positive compartment also distinguish it from other intracellular compartments. The presence of a novel compartment with endosomal/lysosomal characteristics may be a feature shared with other cells that perform specialized functions in connection with membrane traffic. Neuronal cells appear to have more than one endosomal pathway³⁷. The endosomes in the neuronal cell body are devoted to housekeeping functions, whereas in axons and nerve terminals endosomes contain proteins responsible for neurotransmitter release. When the synaptic vesicle protein synaptotagmin is expressed in fibroblasts, it is targeted to a specialized non-housekeeping endosome which is enriched in transferrin receptors for example³⁸. The MHC class II compartment may represent a similar specialization for class II-positive antigen-presenting cells. □

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High birth velocities of radio pulsars

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NEUTRON stars are usually born during the supernova explosion of a massive star. Any small asymmetry during the explosion can result in a substantial 'kick' velocity¹ to the neutron star. Pulsars (rapidly rotating, magnetized neutron stars) have long been known to have high space velocities^{2,3}, but new measurements of proper motion⁴⁻⁶, adoption of a new distance scale for the pulsars⁷ and the realization that some previous velocities were systematically low by a factor of 2 (ref. 8) have prompted us to reassess these velocities. Here, taking into account a strong selection effect that makes the observed velocities unrepresentative of those acquired at birth⁹, we arrive at a mean pulsar birth velocity of $450 \pm 90 \text{ km s}^{-1}$. This exceeds the escape velocity from binary systems, globular clusters and the Galaxy, and so will affect our understanding of the retention of neutron stars in these systems. Those neutron stars that are retained by the Milky Way will be distributed more isotropically than has been thought¹⁰⁻¹², which may result in a distribution like that of the γ -ray burst sources.

Estimates of pulsar transverse velocities V_t have been based primarily on the measurement of proper motion μ (mas yr^{-1}) and the distance D (kpc): $V_t = 4.74 \mu D \text{ km s}^{-1}$. There are 87 reported determinations of proper motion made by interferometry³⁻⁶. We use all these data, apart from pulsar B0736-40 (ref. 6) which lies behind the Gum nebula and has an unknown distance in excess of 0.5 kpc. The distance D is obtained from the new electron density model of Taylor and Cordes⁷ which is based on a number of new independent distance measurements and clearly shows that previous models had underestimated the distance to nearby pulsars. The velocities of pulsars can also be estimated from observations of the speed of the interstellar scintillation patterns^{9,13}. However, Harrison and Lyne⁸ have recently shown that these are systematically low by an average factor of 2 because of a localization of the scattering medium close to the galactic plane. For this reason, we have relied, where possible, on the more direct proper motion measurements. For 14 pulsars which only have upper limits to their proper motion, and a further 13 which do not have proper motion data, we have used scintillation data^{9,14} to estimate V_t .

These scintillation speeds have been calculated using the new distance model⁷ and the appropriate correction for the medium localization⁸. Thus our sample contains 99 pulsars, of which only 8 have upper limits to V_t .

This sample has a mean V_t of $300 \pm 30 \text{ km s}^{-1}$, compared with 134 km s^{-1} obtained 12 years ago from 26 measurements³. The increase in value reflects both the change in the adopted distance scale⁷ and the greater number of young and higher velocity pulsars observed in the recent astrometric surveys⁴⁻⁶. The much improved statistics also show that this velocity is not representative of the velocities at birth because we know that young pulsars have higher velocities on average than older ones⁴. This can be seen in Fig. 1, where the transverse speed of pulsars is plotted against characteristic age, and surely does not arise because pulsars slow down significantly as they age. Rather, it is due primarily to a selection effect, first recognised by Cordes⁹: young pulsars are born from Population I stars, close to the galactic plane, and those pulsars with high velocity ($1,000 \text{ km s}^{-1} \approx 1 \text{ kpc Myr}^{-1}$) mostly move rapidly away from the plane. After $\sim 10 \text{ Myr}$, the mean distance of the fastest pulsars from an observer on the galactic plane is significantly greater than their mean distance at birth, reducing their likelihood of detection and hence lowering the apparent mean velocity. By this time, the pulsars remaining within detectable range are those with small velocity and a few high velocity ones moving roughly parallel to or towards the plane of the Galaxy⁴.

During the first 3 Myr, the strength of the above selection effect is minimal, and the observed transverse speed distribution of the 29 pulsars younger than this should be a good representation of the true V_t distribution at birth. This distribution has a mean of $345 \pm 70 \text{ km s}^{-1}$ and an r.m.s. of 499 km s^{-1} . The mean velocity of only $105 \pm 25 \text{ km s}^{-1}$ for the 10 oldest pulsars illustrates the strength of the effect.

The three-dimensional (3D) space velocities are of course somewhat greater than the observed two-dimensional (2D) transverse velocities, and we can estimate these statistically if we make the reasonable assumption that the velocities at birth are isotropic. We have used a simple, iterative Monte Carlo technique to determine the one-dimensional (1D) velocity distribution required to give the observed 2D transverse distribution, and hence to generate the 3D space velocity distribution. To approximate the observed 2D distribution, we found that the data are well described by a function of the form $x^m/(1+x^n)$, where $x = V_t/V_0$. The scaling factor V_0 and the constants m and n were found to be 330 km s^{-1} , 0.13 and 3.3 respectively. A cumulative plot of this function is compared with the observed

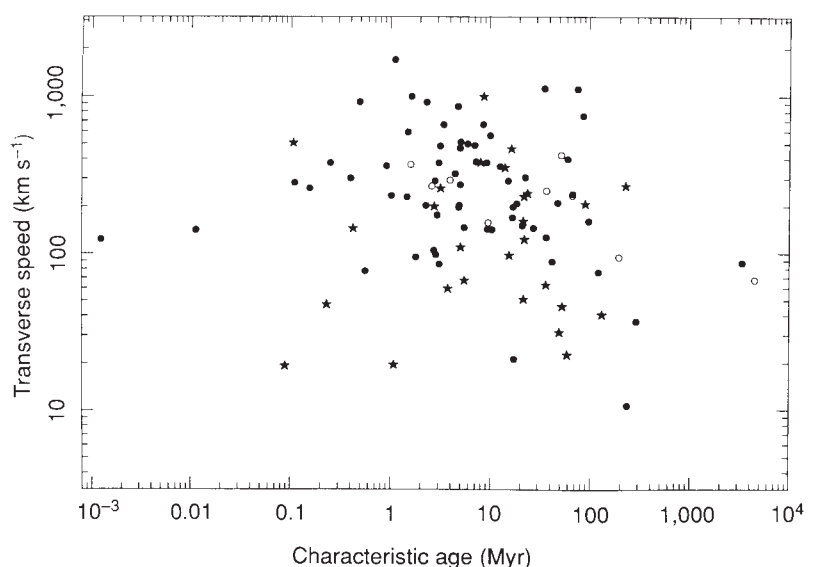
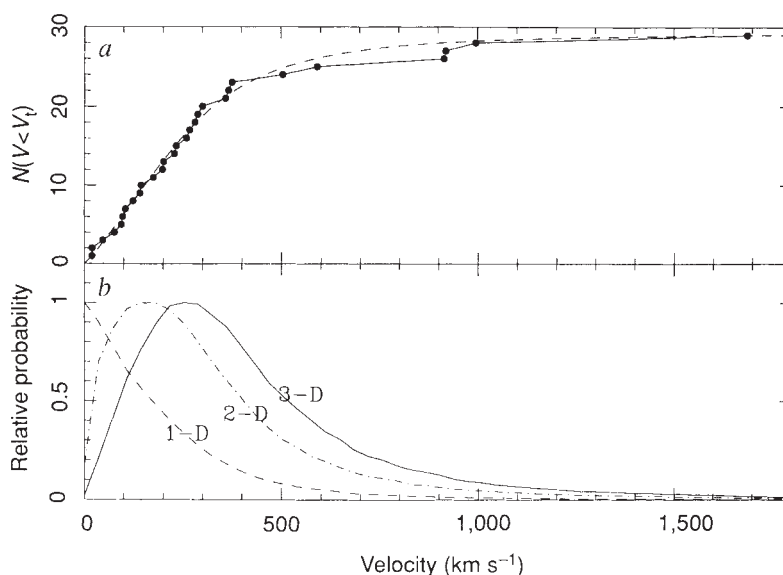


FIG. 1 The transverse speed V_t of 99 pulsars plotted against characteristic age $\tau = P/2\dot{P}$, where P and $\dot{P}$ are the pulsar rotation period and time derivative, respectively. Pulsars with only upper limits to their transverse speed are represented by open circles at half the upper limits. Starred symbols represent transverse speeds obtained from scintillation data. The mean transverse speed for the 29 pulsars younger than 3 Myr is 345 km s^{-1} . In the unlikely circumstance that the two young pulsars in this group with upper limits have zero speed, this value would only be reduced by 6%.

FIG. 2 *a*, The cumulative distribution of transverse speeds V_t for the 29 pulsars younger than 3 Myr. For these pulsars, the selection effect (see text) against detecting high-velocity pulsars is negligible and this is a good representation of the 2D speed distribution at birth. The dashed curve is an analytic approximation to the data having the same mean and r.m.s. (see text). *b*, The one-, two- and three-dimensional velocity distributions corresponding to the analytic function in *a*, obtained using an iterative Monte Carlo simulation. The 3D distribution shows that pulsars have a mean space velocity of $\sim 450 \text{ km s}^{-1}$.



data in Fig. 2*a*, and the derived distributions are shown in Fig. 2*b*. From the 3D distribution, we deduce that the space velocity of pulsars at birth has a mean of $450 \pm 90 \text{ km s}^{-1}$ and an r.m.s. value of 535 km s^{-1} . This mean space velocity is a factor of three higher than the value of $\sim 150 \text{ km s}^{-1}$ deduced from the earlier sample³. The increase arises from the three main factors described above; namely a younger, faster sample ($\times 1.4$), the new distance model ($\times 1.6$) and the selection effect ($\times 1.2$).

There are two other indications that the birth velocities are large. First, such high birth velocities will give rise to a rapid migration from the galactic plane with a mean velocity V_z equal to 210 km s^{-1} , the mean of the 1D distribution in Fig. 2*b*. This is clearly seen for the whole observed pulsar population in Fig. 3 as an increase in the mean distance Z of pulsars from the galactic plane with age. Assuming that pulsars are born in a progenitor Population I distribution with a width $Z_0 = 80 \text{ pc}$, then pulsars of age τ will have a mean height given approximately by $Z = (Z_0^2 + V_z^2 \tau^2)^{1/2}$. This function is also shown in Fig. 3 and describes the data well for ages below about 2 Myr, confirming the large velocity dispersion. For older pulsars, the selection effect described earlier reduces the detected number of large- Z pulsars and hence the mean Z of the observed population.

Second, the high transverse velocities found here are close to

estimates of the velocities of young pulsars required by associations of pulsars with supernova remnants¹⁵. These are usually made on the basis of similarity of age, distance and position on the sky. Often the pulsar has moved significantly from the centre of the remnant since the supernova explosion, so that the pulsar velocity can be determined from the ratio of the separation and the age. Taking 13 reasonably convincing associations, we find that the mean transverse pulsar velocity is $\sim 530 \pm 180 \text{ km s}^{-1}$. Using the same velocity distribution as above, this implies a mean space velocity of $690 \pm 230 \text{ km s}^{-1}$.

We have shown that most pulsars are born with velocities of $\sim 450 \text{ km s}^{-1}$. These large values indicate that their origin must lie in the kinetics of asymmetric supernova collapse rather than the disruption of binary systems^{16,17}. The kinetic energy of the neutron stars so formed are an order of magnitude greater than previously thought. Although the mechanisms of such collapse are not well understood, the high degree of asymmetry now required might also be expected to show corresponding asymmetry in the gaseous supernova remnant in the opposite direction to the pulsar velocity.

Although most recent statistical studies^{18,20} of the pulsar population and its evolution have taken account of the spatial separation of fast and slow pulsars, our results show that this

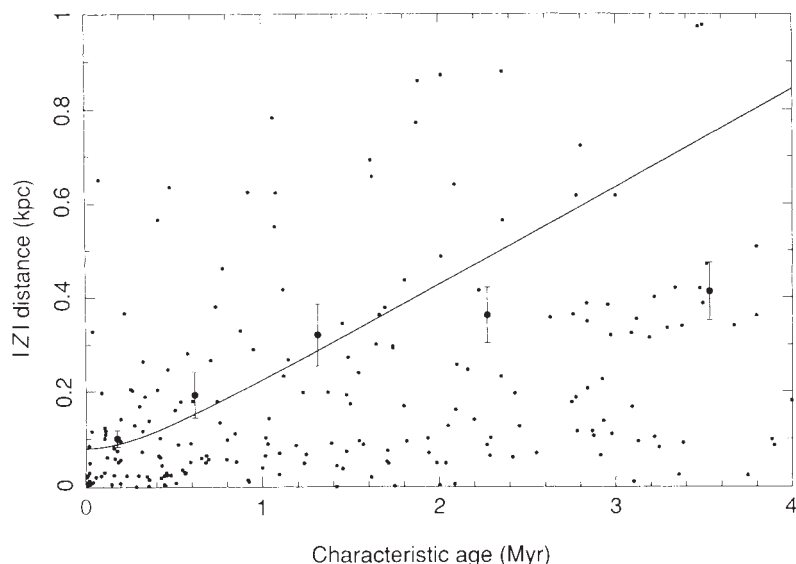


FIG. 3 The distance of pulsars from the galactic plane $|Z|$ shown as a function of characteristic age τ for all the pulsars detected in the major pulsar surveys. The large symbols represent the means of groups of 40 pulsars. The line shows the variation expected from a simple dynamical model with the mean velocity derived from Fig. 2.

has been underestimated by a factor of 3 and they will need to be repeated. Similarly, the increase in birth velocity will clearly have a major effect upon our understanding of the number of pulsars that remain bound in binary systems, globular clusters and the Galaxy. Although such high velocities make it easier to understand the small number of pulsars in binary systems²¹, the small proportion ($\sim 0.7\%$) with velocities below $\sim 50 \text{ km s}^{-1}$ make the large population of neutron stars in globular clusters surprising unless they are formed in a less violent manner, such as accretion-induced collapse of a white dwarf²².

With mean birth velocities of 450 km s^{-1} , more than half of all pulsars will escape the galactic gravitational potential. Those that remain bound will form a much larger spherical halo of old neutron stars than previous models suggested¹⁰⁻¹². Given that the Burst and Transient Source Experiment²³ is continuing to observe an isotropic distribution of γ -ray bursts, our results clearly have a bearing on the possibility that old, high velocity pulsars are the source of the bursts^{24,25}. $\square$

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Relation between fractal dimension and spatial correlation length for extensive chaos

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SUSTAINED nonequilibrium systems can be characterized by a fractal dimension $D \geq 0$, which can be considered to be a measure of the number of independent degrees of freedom¹. The dimension D is usually estimated from time series² but the available algorithms are unreliable and difficult to apply when D is larger than about 5 (refs 3, 4). Recent advances in experimental technique⁵⁻⁸ and in parallel computing have now made possible the study of big systems with large fractal dimensions, raising new questions about what physical properties determine D and whether these physical properties can be used in place of time-series to estimate large fractal dimensions. Numerical simulations⁹⁻¹¹ suggest that sufficiently large homogeneous systems will generally be extensively chaotic¹², which means that D increases linearly with the system volume V . Here we test an hypothesis that follows from this observation: that the fractal dimension of extensive chaos is determined by the average spatial disorder as measured by the spatial correlation length ξ associated with the equal-time two-point correlation function—a measure of the correlations between different regions of the system. We find that the hypothesis fails for a representative spatiotemporal chaotic system. Thus, if there is a length scale that characterizes homogeneous extensive chaos, it is not the characteristic length scale of spatial disorder.

If a large homogeneous nonequilibrium system is extensively chaotic, we can relate its fractal dimension D (roughly the minimum number of degrees of freedom needed to describe a system¹) to its characteristic size L by the equation:

$$D = (L/\xi_c)^d \quad \text{for } L \gg \xi_c \quad (1)$$

where ξ_c is defined to be the chaos correlation length¹² and d is

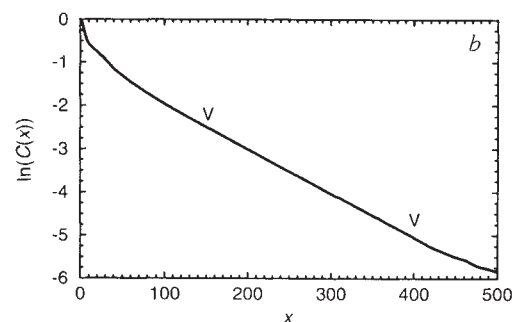
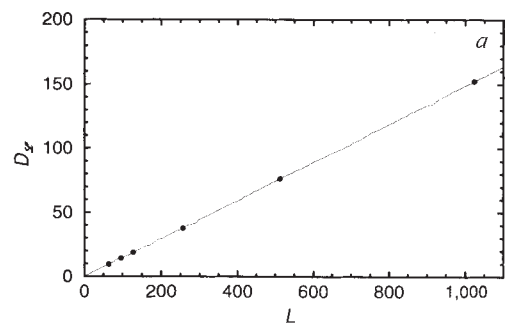


FIG. 1 a, Plot of the Lyapunov fractal dimension D_L against system size L for equation (4) with periodic boundary conditions. We used the parameter values $c_1 = 3.5$ and $c_3 = 0.8$, a constant time step of $\Delta t = 0.05$, a spatial resolution of two Fourier modes per unit length and a total integration time of $T = 50,000$ time units. The error in each fractal dimension is smaller than the size of the plotted points. b, Log-linear plot of the magnitude of the spatial correlation function $C(x) = \langle u^*(x+x', t)u(x', t) \rangle$ for spatiotemporal chaotic solutions of equation (4) for the parameter values $c_1 = 3.5$, $c_3 = 0.81$ and $L = 8,192$. After integrating for a time $T = 30,000$ to allow transients to decay, the correlations were calculated over an interval of 70,000 time units. The correlation function was further averaged over 64 different initial conditions. The two Vs indicate the region over which an exponential decay was identified. There is no change in the correlation function for larger system sizes.

the spatial dimensionality of the system, for example, $d=2$ for a large-aspect-ratio convection experiment⁵. Equation (1) can be understood as a consequence of spatial disorder: parts of a large system will be uncorrelated and hence dynamically independent when separated by distances larger than ξ_c . The volume $V=L^d$ of the system then acts as a gas of L^d/ξ_c^d weakly-interacting regions of volume ξ_c^d which contribute independently to the fractal dimension. The assumption of extensive chaos, that $D \propto L^d$ in equation (1), has been confirmed in several numerical calculations⁹⁻¹¹ and is believed to hold generally for large chaotic systems for reasons first given by Ruelle¹³.

The interesting question then arises as to what the chaos correlation length ξ_c means, or what the small volume ξ_c^d corresponds to. If it turns out that one can estimate ξ_c from spatiotemporal measurements, then equation (1) could be used directly to find the fractal dimension of high-dimensional systems, bypassing the well-known problems of time-series algorithms^{2,3}. As an example, we note that the two-point correlation function has been used to estimate a correlation length $\xi \approx 6$ mm from voltage measurements on the surface of a fibrillating pig heart¹⁴. Because the radius of the heart was $R \approx 25$ mm, equation (1) might suggest (assuming $\xi_c \approx \xi$) that the fibrillating heart involved $D \approx 4\pi R^2/\pi \xi^2 \approx 70$ independent regions, a result that is consistent with the failure of time-series algorithms to detect low-dimensional chaos¹⁵.

In the following, we test the simple hypothesis that the chaos correlation length ξ_c is proportional to the most commonly used measure of spatial disorder, the correlation length ξ obtained from exponentially-decaying two-point equal-time correlation functions (see Fig. 1):

$$\xi_c \propto \xi \quad (2)$$

(We note that not all spatiotemporal chaotic systems have exponential decay of spatial correlations, for example, systems with conservation laws can have algebraically decaying correlation functions corresponding to an infinite ξ ¹⁶.) To obtain an equation that can be falsified, we observe that equation (1) predicts

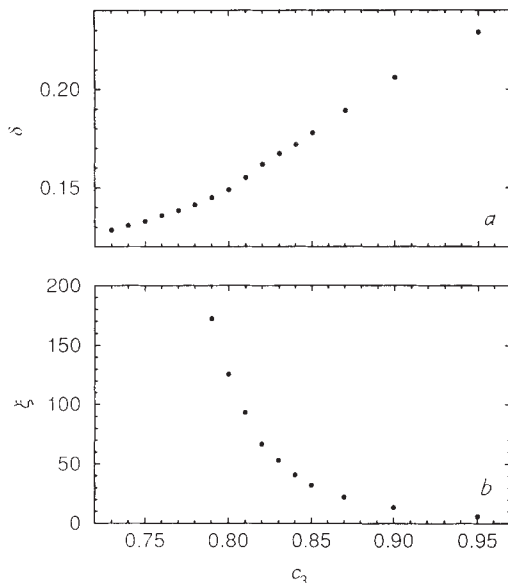


FIG. 2 a, Plot of the Lyapunov dimension density δ against the parameter c_3 for fixed $c_1 = 3.5$. Dimension densities were obtained from the slopes of figures similar to Fig. 1a. To within numerical accuracy, the curve is continuous across the L_1 transition region²⁰. b, Plot of the spatial correlation length ξ against the parameter c_3 for fixed $c_1 = 3.5$. The ξ values were obtained by least-squares fits to the linear portion of correlation plots similar to Fig. 1b.

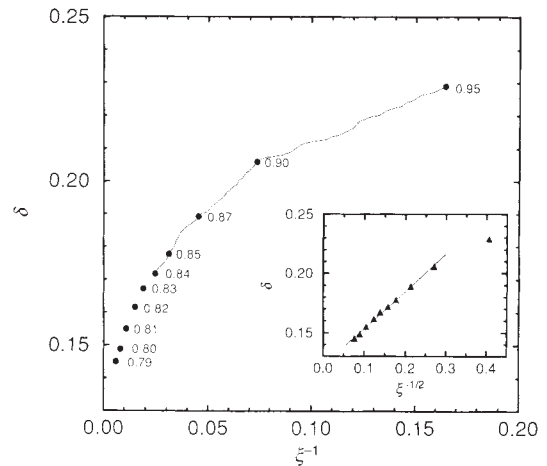


FIG. 3 Plot of the Lyapunov dimension density δ against the inverse correlation length $1/\xi$ for the parameter value $c_1 = 3.5$. The different values of the parameter c_3 are shown next to each point. The inset shows the same data plotted against $1/\sqrt{\xi}$. The dotted lines connecting adjacent points were added to guide the eye in identifying linear trends. The nonlinear dependence on $1/\xi$ is inconsistent with the scaling law equation (1) for a gas of weakly coupled domains that are highly correlated over the range of the correlation length ξ .

that $D \propto 1/\xi_c^d$ for fixed system size L , that is, a smaller chaos correlation length implies a larger fractal dimension. It is useful to express this relation in terms of an intensive quantity so that the size L and details of the boundary conditions no longer enter. This can be done by introducing the intensive density $\delta = D/L^d$, which is the fractal dimension per unit volume for an extensively chaotic system. (For a particular model, Torcini *et al.*¹⁷ have shown in Fig. 10 of their paper that δ is, indeed, independent of the choice of boundary conditions for sufficiently large system sizes.) Equations (1) and (2) then imply that

$$\delta \propto 1/\xi^d \quad (3)$$

which we test below.

To investigate the dependence of the dimension density δ on the correlation length ξ , we have studied chaotic numerical solutions of the one-dimensional complex Ginzburg-Landau equation¹²:

$$\partial_t u(x, t) = u + (1 + ic_1)\partial_x^2 u - (1 - ic_3)|u|^2 u \quad (4)$$

in a periodic system of size L , where x is the spatial coordinate and t is time. The field $u(x, t)$ is complex-valued, whereas the parameters c_1 and c_3 are real-valued. Equation (4) is an experimentally relevant^{18,19} continuum dynamical model that holds universally near the onset of a Hopf bifurcation from a static homogeneous state to an oscillatory state¹². For $c_1 \geq 1.8$, equation (4) is particularly useful for testing equation (3) as there is a strong variation of ξ with parameter c_3 near the so-called L_1 transition separating phase- from defect-chaotic states²⁰. The correlation length ξ increases rapidly as the L_1 transition is approached from the defect-chaos side although it does not diverge.

We integrated equation (4) using a pseudospectral method with time-splitting of the operators²¹, carefully checking our results for convergence with respect to spatial and temporal resolutions and with respect to total integration times. Correlation lengths ξ were obtained by examining the asymptotic exponential decay $A \exp(-x/\xi)$ of the magnitude of the complex two-point equal-time correlation function $C(x) = \langle u^*(x+x', t)u(x', t) \rangle$, where the angle brackets indicate averaging over time t , space x' and realizations. We calculated a particular fractal dimension, the Lyapunov dimension D_∞ , by the

Kaplan-Yorke formula which expresses $D_{\mathcal{Y}}$ in terms of the spectrum of Lyapunov exponents²². The exponents were obtained by an expensive but widely-used algorithm²² that involves integrating equation (4) together with many copies of the linearization of this equation around the solution u . We used about 1,500 hours on CRAY YMP and on Thinking Machines CM-5 supercomputers to obtain the results reported below. For most dynamical systems, the Lyapunov dimension is believed to be the same as the information dimension D_1 , which is just one of an infinity of fractal dimensions D_q that characterize multifractal strange attractors¹.

Our results are presented in Figs 1–3 for the fixed parameter $c_1 = 3.5$ and for various values of the parameter c_3 near the L_1 transition. We have calculated correlation lengths ξ for various system sizes up to $L = 262,144$, and Lyapunov dimensions $D_{\mathcal{Y}}$ for system sizes only up to $L = 1,024$ (as the latter is far more expensive to calculate numerically). Figure 1a confirms the assumption of extensive chaos in equation (1) with $d=1$ by showing that $D_{\mathcal{Y}}$ grows linearly with system size L over the entire range $64 \leq L \leq 1,024$. For $L = 1,024$, we estimate a large fractal dimension of $D \approx 152$. We estimated the dimension density δ by a least-squares estimate of the slope of a linear portion of this curve; this linear portion does not extrapolate to zero dimension when $L = 0$. Similar extensive chaos curves were obtained for all c_3 values reported here. Figure 1b shows how we calculated the correlation length ξ from the asymptotic exponential decay of the spatial correlation function $C(x)$. It is interesting to observe that the extensive chaos regime in Fig. 1a commences for a system size $L \approx 50$ that is smaller than ξ .

Figure 2 summarizes how the Lyapunov dimension density and spatial correlation length vary with the parameter value c_3 for the particular value $c_1 = 3.5$. As c_3 decreases through the L_1 transition region ($c_3 \approx 0.74$), the Lyapunov dimension density (Fig. 2a) decreases continuously within our numerical accuracy. The correlation length ξ has contrary behaviour and increases by a factor of 30 close to the L_1 transition (from 6 to 172). Figure 3 presents the data of Fig. 2 in a more appropriate way for testing equation (3). For spatial dimension $d=1$, the dimension density δ should be linear in the quantity $1/\xi$ whereas, in fact, the observed dependence is unambiguously nonlinear. The inset figure suggests that a $1/\sqrt{\xi}$ dependence may be more appropriate.

We conclude that equation (2) is not correct: the chaos correlation length is not simply related to the two-point correlation length and so the fractal dimension is not related to spatial disorder as measured by the most commonly used correlation length. Calculations (C. O'Hern, D.A.E. and H.S.G., unpublished results) on a two-dimensional coupled map lattice that undergoes a nonequilibrium Ising transition²³ give a similar result: δ (and so ξ_c) varies smoothly and is finite at the Ising transition point while ξ diverges to infinity so that equation (2) cannot hold. Further analysis will be needed to answer the important question of the geometric meaning of the chaos correlation length and the related question of whether this length scale (and hence the fractal dimension) can be estimated by methods that are more direct and efficient than time-series methods² or calculation of the Lyapunov spectrum from known equations of motion²². □

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Self-organized growth of strained InGaAs quantum disks

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LATERAL confinement of electrons or excitons in low-dimensional semiconductor structures (quantum 'wires' and 'boxes') leads to new electronic properties¹, which can be used to improve the performance of optical devices such as semiconductor lasers and nonlinear optical switches^{2–4}. Several state-of-the-art technologies have been applied to fabricate these quantum structures: lateral structure can be defined using high-resolution lithography combined with dry etching^{5,6}, or by crystal growth on masked substrates^{7,8}. Unfortunately, such processes inevitably result in a deterioration of the crystal quality. But recent reports^{9,10} of self-organized formation of quantum-wire semiconductor structures have attracted considerable interest as a means of overcoming these difficulties. We describe here the self-organized formation of box-like microstructures during the interrupted epitaxial growth of strained InGaAs/AlGaAs multilayer structures on (311)B gallium arsenide substrates. We find that the InGaAs layers organize spontaneously into homogeneous nanoscale disks embedded in an AlGaAs matrix. This phenomenon appears to arise from a complex interplay between the lattice strain, surface energy and surface migration.

The samples studied here were grown in a vertical low-pressure metal organic vapour-phase epitaxy (MOVPE) reactor using arsine and the trimethyl alkyls of gallium (TMG), aluminum (TMA) and indium (TMI) source gases. The growth rate was $0.6 \mu\text{m h}^{-1}$ for $\text{In}_z\text{Ga}_{1-z}\text{As}$ and $1.2 \mu\text{m h}^{-1}$ for $\text{Al}_x\text{Ga}_{1-x}\text{As}$, the group-V-to-III flux ratio was 100:1, and the total H_2 flux was 5 standard l min^{-1} . The epitaxial layer thickness and In content have been calibrated for thick layers on conventional (100) GaAs substrates by cross-sectional scanning electron microscopy (SEM) and X-ray diffraction, respectively. The layer thickness was controlled by the growth time interval using mass-flow controllers, and confirmed by SEM to be equal (in particular) for (100) and (311)B oriented $\text{Al}_x\text{Ga}_{1-x}\text{As}$ layers.

The growth mode of (311)B $\text{In}_z\text{Ga}_{1-z}\text{As}$ was investigated in the following layer sequence grown at a temperature of 800°C . Three $\text{In}_z\text{Ga}_{1-z}\text{As}$ layers with nominal In content of $z = 0.2$ and thickness of 5 nm (confirmed on (100) reference substrates) were grown and separated by 30-nm-thick $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$ barriers. The growth interruption time between the layers was 10 s. After the third $\text{In}_z\text{Ga}_{1-z}\text{As}$ layer, a 100-nm-thick upper $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$ barr-

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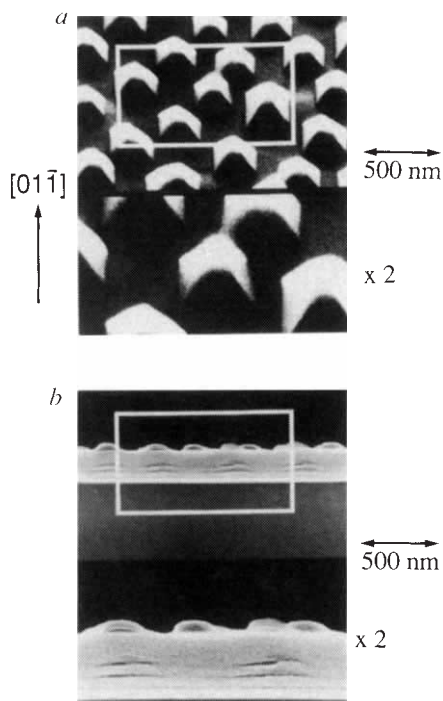


FIG. 1 *a*, Scanning electron topographical micrograph of nominal 10-nm (311)B $\text{In}_{0.2}\text{Ga}_{0.8}\text{As}$, forming quantum disks embedded in $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$ microcrystals. *b*, Cross-sectional image of the same structure. (The arrow-headed scale bar applies to the top half of *a* and *b*. The bottom half in each case is at 2 $\times$ this magnification.)

ier was grown, and finally capped with nominal 10-nm $\text{In}_{0.2}\text{Ga}_{0.8}\text{As}$. After growth, the substrate heating was stopped and the samples were cooled at a rate of $\sim 20^\circ\text{C min}^{-1}$.

The SEM topographical image of the nominal 10-nm-thick $\text{In}_{0.2}\text{Ga}_{0.8}\text{As}$ layer (Fig. 1*a*) reveals the formation of an array of well-ordered microcrystals with a clearly faceted surface. The surprising alignment of the microcrystals suggests a strong positional correlation during the formation process related to the appearance of the facet planes. The formation mechanism and composition of the microcrystals is clarified by the cross-sectional image obtained after stain etching (Fig. 1*b*). The microcrystals are found to be composed of $\text{In}_z\text{Ga}_{1-z}\text{As}$ islands with a diameter of 150-nm, embedded in an $\text{Al}_x\text{Ga}_{1-x}\text{As}$ matrix with a thickness of ~ 30 nm. Note that no $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$ has been grown after the deposition of the $\text{In}_z\text{Ga}_{1-z}\text{As}$ layer. This implies that after the growth, the $\text{In}_z\text{Ga}_{1-z}\text{As}$ film first forms islands that are then buried by $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$, which was provided by lateral mass transport from the buffer layer (no contrast is observed between the $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$ buffer layer and the $\text{Al}_x\text{Ga}_{1-x}\text{As}$ microcrystal coating the disk). We therefore consider that the shape of the $\text{In}_z\text{Ga}_{1-z}\text{As}$ islands is accommodated to that of the microcrystals during the coating to form the final quantum disk. In addition, redistribution of In is expected which will be discussed below.

The dynamics of the process is further revealed in the evolution of the nominal 5-nm-thick $\text{In}_{0.2}\text{Ga}_{0.8}\text{As}$ quantum wells in the buffer layer, grown with 10-s growth interruption. The flatness of the first quantum well suggests that islanding and mass transport takes place on a timescale between several seconds and several minutes. However, the disk-like morphology of the second and third $\text{In}_{0.2}\text{Ga}_{0.8}\text{As}$ film suggests enhancement of the formation rate due to surface waves introduced by strained layer overgrowth¹¹. Increasing the nominal In content (z) and decreasing the nominal $\text{In}_z\text{Ga}_{1-z}\text{As}$ layer thickness (d) allows us to alter

the average size (measured across the contrast at the base) and separation distance of the microcrystals in a reproducible manner from 210 nm for $z=0.2$ and $d=10$ nm, down to 70 nm for $z=0.5$ and $d=3$ nm, which corresponds to a $\text{In}_z\text{Ga}_{1-z}\text{As}$ disk size of ~ 30 nm. Figure 2*a* shows the nominal 3-nm $\text{In}_{0.3}\text{Ga}_{0.7}\text{As}$ layer and Fig. 2*b* shows the nominal 3-nm $\text{In}_{0.5}\text{Ga}_{0.5}\text{As}$ layer. Therefore, the nominal layer thickness d mainly determines the areal density of the microcrystals rather than their size. As the size of the microcrystals is decreased, their positional correlation becomes less pronounced; however, their size distribution remains almost unchanged, well within 10% of the average size. Finally, for our $\text{In}_{0.2}\text{Ga}_{0.8}\text{As}$ layers the disk size is found to be almost independent of the Al content x of the buffer layer for x -values < 0.7 . For higher Al contents, the formation of the microcrystals is hindered due to the small surface diffusion length of Al.

The observed process of microcrystal formation may be understood by considering the complex interplay of surface energy, strain and surface migration. Due to the lattice mismatch (of 0–7.2%) between $\text{In}_z\text{Ga}_{1-z}\text{As}$ and $\text{Al}_x\text{Ga}_{1-x}\text{As}$ (for $z=0$ –1), epitaxial layers of $\text{In}_z\text{Ga}_{1-z}\text{As}$ on GaAs are highly strained. The total strain energy of $\text{In}_z\text{Ga}_{1-z}\text{As}$ films is reduced by the formation of coherent islands (that is, without dislocations) due to the elastic relaxation at the free edges of the islands, which outweighs the cost of additional surface energy^{12,13}. But at the early stage of growth, where the energy-lowering due to the formation of islands is small, the flat morphology is usually maintained due to a high energy barrier to surface migration¹⁴. On (311)B planes the surface energy is initially high¹⁵, increasing the instability to thermal roughening which is combined with a high surface migration. Thermal roughening provides nucleation sites for ‘islanding’, which can be supported for the undulated growth front after strained layer overgrowth. The reduction of the size and separation distance of the microcrystals with increasing In content z is attributed to the reduced $\text{In}_z\text{Ga}_{1-z}\text{As}$ island size at higher strain energies, due to the increasing energy difference between an island film and a flat film at higher strain¹⁶. Finally, a high surface mobility allows the immediate coverage of the $\text{In}_z\text{Ga}_{1-z}\text{As}$ islands with material from the buffer layer due to lateral mass transport. The driving force for the mass transport toward the islands is assumed to be a reduction of surface strain energy of the (strained) $\text{In}_z\text{Ga}_{1-z}\text{As}$ islands on formation of the $\text{Al}_x\text{Ga}_{1-x}\text{As}$ microcrystals lattice, which is matched to the buffer with an unstrained surface. The microcrystals are also expected to expose low-index facets of low surface energy¹⁷.

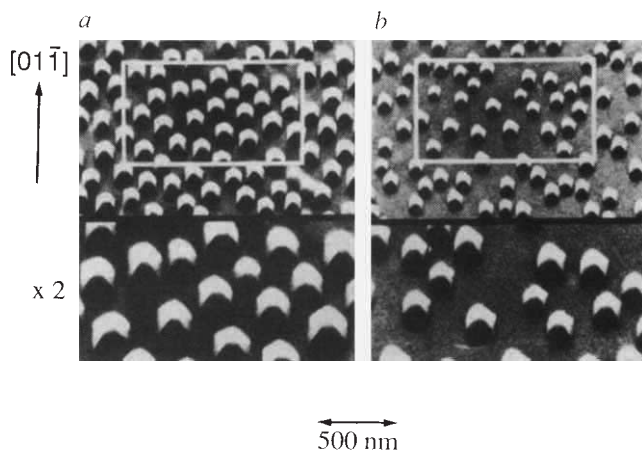


FIG. 2 Scanning electron topographical micrograph of *a*, nominal 3-nm (311)B $\text{In}_{0.3}\text{Ga}_{0.7}\text{As}$, and *b*, nominal 3-nm (311)B $\text{In}_{0.5}\text{Ga}_{0.5}\text{As}$, forming quantum disks with a diameter of 30 nm. Note that the scale in *a*, *b* and Fig. 1*a* is the same. (The arrow-headed scale bar applies to the top half of *a* and *b*. The bottom half in each case is at 2 $\times$ this magnification.)

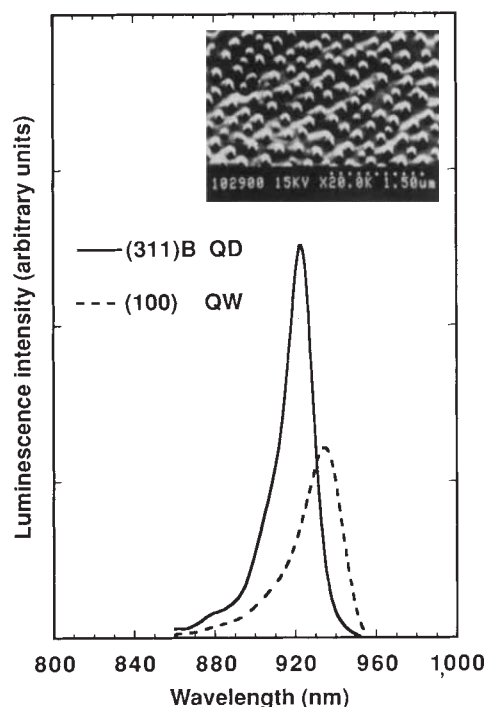


FIG. 3 Room-temperature (300 K) photoluminescence of the nominal 6-nm $\text{In}_{0.25}\text{Ga}_{0.75}\text{As}$, forming quantum disks (QD) with a diameter of 80 nm, and of the (100) reference quantum well (QW). (Power density of laser excitation is 10 W cm^{-2} .) Inset, Scanning electron topographical micrograph of the microcrystals coating the disks without overgrowth. (Dotted scale bar, $1.50 \mu\text{m}$.)

The solid curve in Fig. 3 shows the photoluminescence (PL) spectrum at room temperature of nominal 6-nm $\text{In}_{0.25}\text{Ga}_{0.75}\text{As}$, formed into disks with a diameter of 80 nm. For excitation the green (488 nm) line of an Ar^+ laser was used with an excitation density of 10 mW cm^{-2} . For the PL measurements the microcrystals shown in the inset in Fig. 3 are buried with 50-nm $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$ after 3 min growth interruption. The $\text{Al}_{0.5}\text{Ga}_{0.5}\text{As}$ barrier contains a nominal 15-nm-thick $\text{In}_{0.2}\text{Ga}_{0.8}\text{As}$ layer to initiate islanding. The weak emission from this layer peaks around 955 nm and does not contribute to the PL spectrum from the disks shown in Fig. 3. The excellent crystal quality of the disks manifests itself as the strong measured integrated PL intensity, which is comparable to that of conventional $\text{In}_x\text{Ga}_{1-x}\text{As}$ quantum wells grown side-by-side on (100) GaAs substrates as reference (dashed curve). Taking into account the filling factor, that is the coverage of the $\text{In}_x\text{Ga}_{1-x}\text{As}$ containing structure, the luminescent intensities of the disks seems to exceed that of the quantum well by more than one order of magnitude. The high uniformity of the disks is evidenced in the small full-width at half-maximum of the luminescence line (19 meV) compared to that of the reference (100) quantum-well structure (32 meV).

It is difficult to determine (by chemical and structural analysis) whether the luminescence line sharpening and the blue shift is due to lateral confinement effects, or has to be attributed to differences in the strain state, the distribution of In, and interface ordering during the self organization. Although the close PL line positions of the disk and reference quantum well tells us that the average In content is comparable, quantitative analysis of this phenomenon requires the precise In composition profile. This will be very complex, largely due to the surface segregation of In during the coating of the $\text{In}_x\text{Ga}_{1-x}\text{As}$ islands. But strain-induced interface ordering due to islanding is a very unusual

phenomenon in itself, with important implications for device applications, and will therefore be the topic of further investigations. Indeed, the PL linewidth of $\text{In}_x\text{Ga}_{1-x}\text{As}$ (311)B quantum wells grown without growth interruption is significantly larger than that of the corresponding disks with longer peak-emission wavelengths. Preliminary results on (211)B and (511)B GaAs surfaces show similar phenomena of self-organization of strained $\text{In}_x\text{Ga}_{1-x}\text{As}$ films, which seems to be a rather common feature of strained-layer growth on high-index substrates. We therefore expect a variety of different structures on other planes and material systems, opening a wide field for investigation. □

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A chemically and electrochemically switchable molecular shuttle

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THE developing field of nanotechnology has generated wide interest across a broad range of scientific disciplines¹. In particular, the realization of nanoscale switching devices might have far-reaching implications for computing and biomimetic engineering^{2–4}. But miniaturization of existing semiconductor technology may not be the best approach to the fabrication of structures whose dimensions are smaller than the wavelength of the radiation used in optical lithography and etching techniques⁵. The approach observed in the natural world, whereby nanostructures are built up through the self-assembly^{6–9} of smaller molecular entities, holds substantial promise. Nature abounds with molecular switching devices which perform a variety of functions, such as the transport of metabolites across cell membranes or the signalling of nerve impulses. These processes are commonly controlled by stimuli such as changes in ion concentrations and electrical potentials. Here we report the synthesis of a supramolecular structure (compound 1·[PF₆]₄, Fig. 1A) that can be reversibly switched between two states by proton

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concentration changes or by electrochemical means. The supermolecule is a rotaxane comprising a molecular ring threaded on an axle containing two 'docking points'. We can effect controlled switching of the ring from one of these positions to the other. We use ^1H NMR and ultraviolet/visible spectroscopy to characterize the dynamics of the bead's movement along the thread before and after switching.

The rotaxane structure resembles a bead trapped on a linear thread which is terminated by two bulky stopper groups¹⁰. The bead can shuttle back and forth along the thread but cannot dissociate because of the presence of the terminal stoppers. Recently, various methods^{11,21} have been developed that permit the template-directed synthesis of rotaxanes in yields much higher than those obtained from the former undirected, statistical methods¹⁰. In the rotaxane (Fig. 1A) that we describe here, the dumb-bell component contains two different π -electron-donor stations, and the bead is a π -electron-accepting tetracationic cyclophane^{22,23} comprising two bipyridinium units bridged by two *p*-xylyl spacers. The dumb-bell component consists of biphenol and benzidine units (as π -electron donors)

inserted within a polyether chain terminated by stoppers in the form of tri-isopropylsilyl groups. The [2]-rotaxane $1\cdot[\text{PF}_6]_4$ was obtained in 19% yield in the final assembly step, using a method similar to that already reported for the template-directed synthesis of rotaxanes^{11,12}.

At room temperature, the bead moves back and forth along the thread at a rate comparable to the NMR timescale. This leads to the extreme broadening of many NMR signals for $1\cdot[\text{PF}_6]_4$ in CD_3CN solution, and precludes the detailed assessment of preferred bead location in the [2]-rotaxane under these conditions. But on cooling this solution to 229 K, we were able to observe (Fig. 2A) distinct signals from two translational isomers, corresponding to the occupation by the macrocyclic bead of the benzidine and biphenol stations. Two-dimensional NMR spectroscopy proved to be a valuable tool in the interpretation of this spectrum, the complexity of which arises partly from the inequivalence of the two phenylene rings in each aromatic station—in both the major and the minor isomers. In the major isomer, the protons giving these signals have been labelled *a-h* (Fig. 1A). Examination of the COSY-90 spectrum only provides

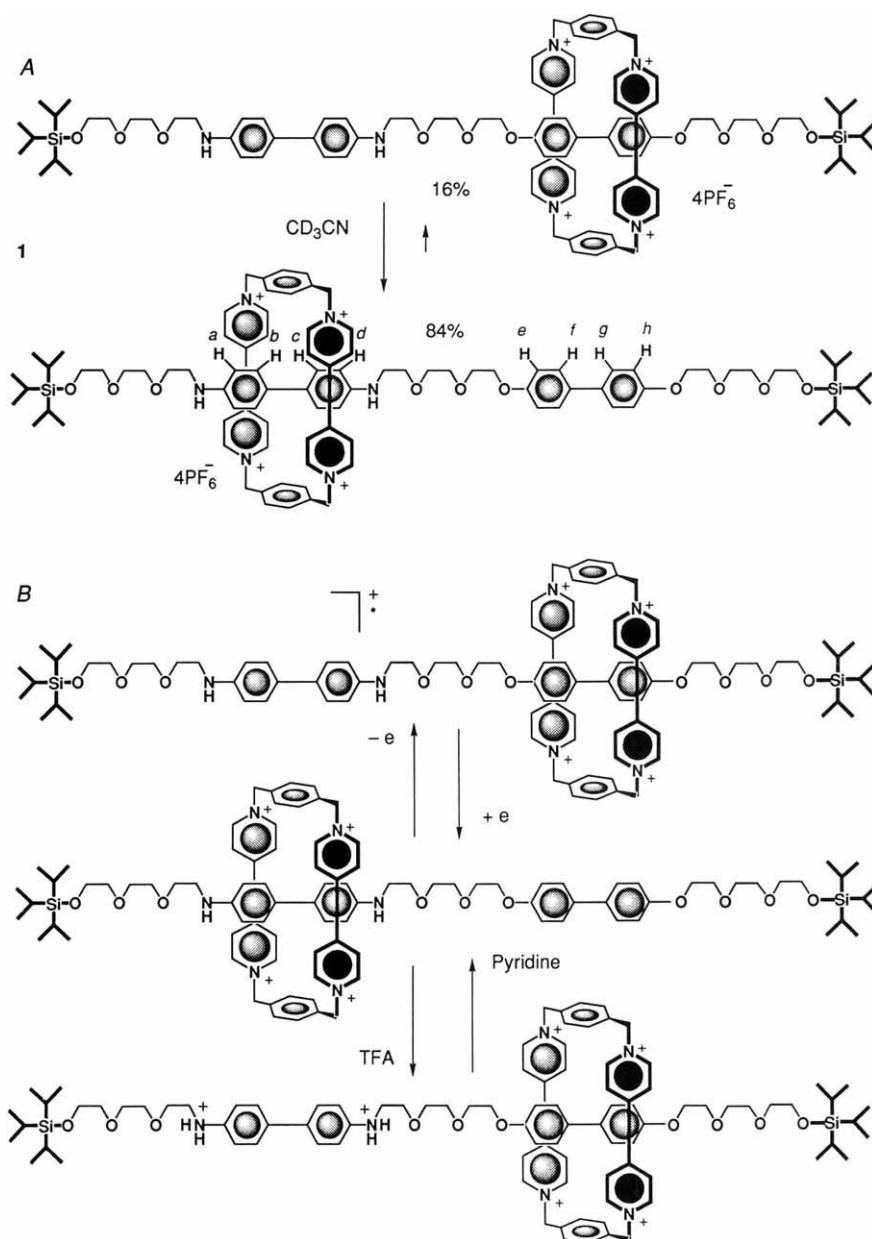


FIG. 1 A, Structure of the switchable rotaxane. At equilibrium in CD_3CN , both stations are occupied, the benzidine station (left) preferentially. B, Protonation (bottom) or electrochemical oxidation (top) of the benzidine station results in switching of the bead so that it resides preferentially over the biphenol (right) station.

information about the major isomer because the low intensity of the signals from the minor isomer fail to produce sizeable cross-peaks. The NOESY spectrum relates signals from the major and minor isomers which are exchanging as a consequence of the bead shuttling process. In this way, we were able to assign the resonances of all the biphenyl protons in each isomer (see details in Fig. 2). Comparison of the chemical shifts for the station which is unoccupied by the cyclophane, in each isomer,

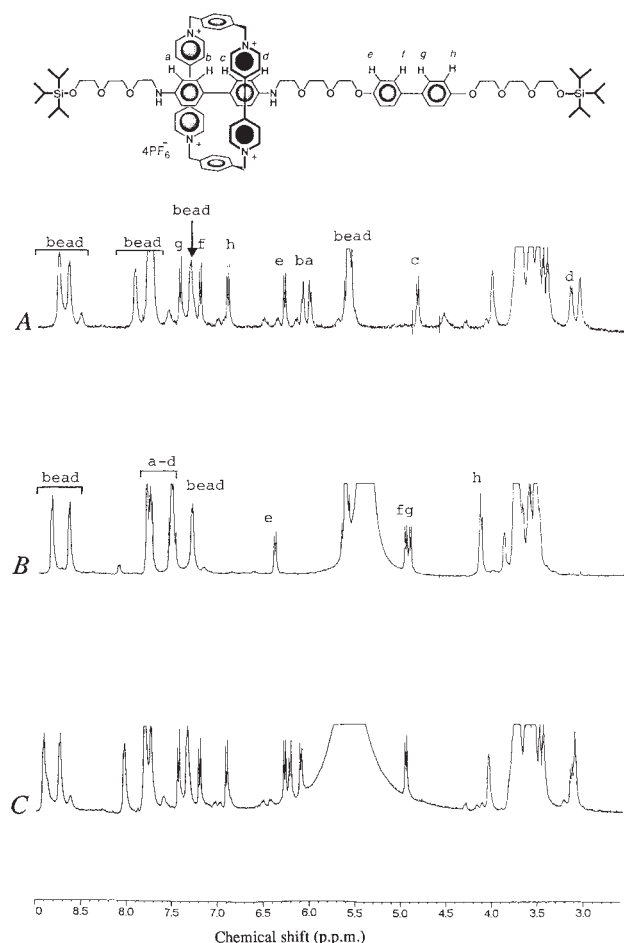


FIG. 2 400 MHz ^1H NMR spectra (229 K, CD_3CN) of samples containing A, 0.46 mM $\mathbf{1}\cdot[\text{PF}_6]_4$; B, 0.46 mM $\mathbf{1}\cdot[\text{PF}_6]_4$ + 55 mM TFA-d_1 ; and C, 0.46 mM $\mathbf{1}\cdot[\text{PF}_6]_4$ + 55 mM TFA-d_1 + 64 mM pyridine- d_5 . Off-scale peaks correspond to protons from the cyclophane bead, residual water or bismethylenedioxy linkages. The resonances resulting from the protons in the cyclophane bead occur at δ 8.78, 8.65, 8.54, 7.94, 7.75, 7.58, 7.33 and 5.59 p.p.m. The assignment of the aromatic protons of the dumb-bell component was based on COSY-90 results. For instance, in A one observes a coupled pair of doublets at δ 3.25 and 4.87 p.p.m.; previous experience with related systems^{11,12} suggests that these two signals arise from one of the phenylene rings of the station which is occupied by the cyclophane bead. The next most upfield pair of coupled doublets occurs at δ 6.03 and 6.11 p.p.m. These signals were assigned to the other phenylene ring of the occupied station. Of the remaining four large doublets, which must correspond to the unoccupied station of the major isomer, those at δ 6.93 and 7.45 p.p.m. exhibit chemical shifts very similar to the proton resonances of the biphenol moiety in the uncomplexed thread molecule, and must correspond to protons *h* and *g*, respectively (shown in the molecular formula at the top of the Figure). In summary, the chemical shifts of the protons in the two stations of the major isomer (before protonation) were assigned as follows (all δ in p.p.m.): *a*, δ 6.03; *b*, δ 6.11; *c*, δ 4.87; *d*, δ 3.25; *e*, δ 6.34; *f*, δ 7.24; *g*, δ 7.45; *h*, δ 6.93. Details of proton assignments on protonation (corresponding to spectrum B) are given in the text.

clearly shows that the biphenol subunit is vacant in the major isomer of $\mathbf{1}\cdot[\text{PF}_6]_4$.

The relative proportions (Fig. 1A) of the two translational isomers were obtained from the integration of the respective ^1H NMR resonances and correspond to 84% occupation of the benzidine station and 16% occupation of the biphenol station (at 229 K). The preferential occupation of the benzidine station was anticipated from previous binding studies¹² which demonstrated that the binding affinity of the cyclophane for a model benzidine derivative is ~ 10 times greater than that for the corresponding biphenol derivative. The average position of the bead can be switched over to the biphenol domain of the thread either by protonation of the basic nitrogens of the benzidine residue or by electrochemical oxidation of this station (Fig. 1B). Both of these means of control over the shuttling process rely on the repulsive electrostatic interactions between the positively charged bead and the post-switching cationic nature of the benzidine station.

Addition of excess deuterated trifluoroacetic acid (TFA-d_1) to the solution of $\mathbf{1}\cdot[\text{PF}_6]_4$ in CD_3CN results in large changes in the NMR spectrum (Fig. 2B). First, only resonances from one translational isomer can be detected, which considerably simplifies the spectrum. The COSY-90 spectrum obtained under these conditions shows a pair of coupled multiplets at δ 7.57 and 7.80 p.p.m. which, by comparison with the spectrum of the free thread under the same acidic conditions, correspond to the aromatic protons (*a-d*) of the protonated benzidine nucleus. More interestingly, however, we observe a set of two coupled doublets (at δ 4.20 and 4.98 p.p.m.) and another set at δ 5.03 and 6.44 p.p.m. corresponding to the protons (*e-h*) of the biphenol station which is not complexed by the cyclophane bead, that is, all the signals that pertain to the biphenol station are now shifted considerably upfield compared with those of the biphenol station in the free dumb-bell.

Neutralization of the added TFA-d_1 with pyridine- d_5 essentially regenerates the spectrum (Fig. 2C) obtained before acid addition. This finding, together with independent chromatographic studies, rules out the possibility of acid-catalysed desilylation of the thread component. Furthermore, this result establishes the reversible nature of the structural changes caused by the protonation/deprotonation of the benzidine residue.

The conclusions obtained from the ^1H NMR spectroscopic studies are supported by room-temperature ultraviolet/visible spectroscopy. We have shown¹² previously that a benzidine derivative forms a green charge-transfer complex with the cyclo-

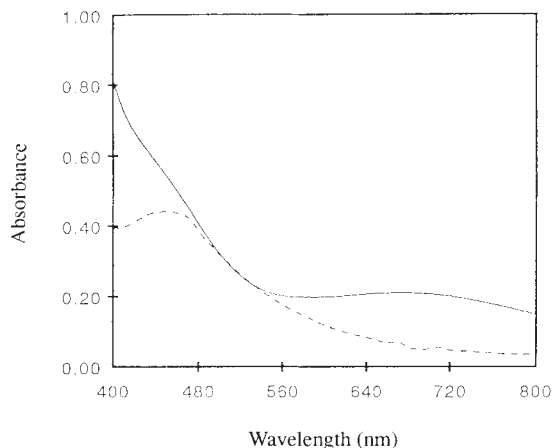
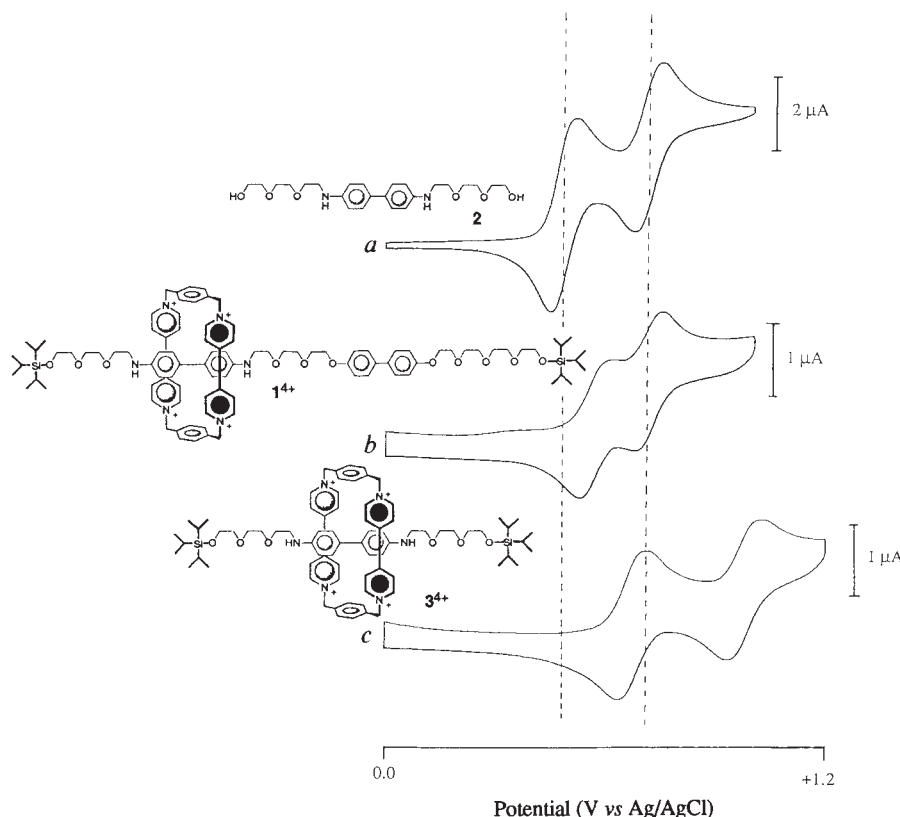


FIG. 3 Visible absorption spectra of an acetonitrile solution containing 0.5 mM $\mathbf{1}\cdot[\text{PF}_6]_4$ in the absence (continuous line) and in the presence (dashed line) of 50 mM TFA. Optical pathlength, 1.0 cm.

FIG. 4 Cyclic voltammetric response on a glassy carbon working electrode of a, 1.0 mM compound **2**; b, 0.5 mM **1** $\cdot$ [PF₆]₄; and c, 0.7 mM **3** $\cdot$ [PF₆]₄. Solvent system, 0.1 M TBA⁺PF₆⁻/CH₃CN. Scan rate, 0.100 V s⁻¹. The vertical dotted lines indicate—for comparison purposes—the position of the half-wave potentials of the two one-electron oxidations of compound **2**.



phane ($\lambda_{\text{max}} = 690$ nm), whereas the analogous biphenol species produces a red complex ($\lambda_{\text{max}} = 480$ nm). At room temperature, the molecular shuttle investigated here exhibits both charge-transfer bands (Fig. 3), indicating that the two stations are partially occupied, but on addition of excess TFA, the band centred at 690 nm disappears. Thus, protonation of the benzidine nitrogen atoms creates an electrostatic barrier which confines the bead around the biphenol station. A more quantitative description of these spectroscopic changes is hindered by tailing ultraviolet absorptions, associated with the benzidine subunit, which prevent the 'clean' observation of the biphenol-cyclophane charge-transfer band. However, the TFA-induced spectroscopic changes can be fully reversed by neutralization of the TFA with added pyridine.

Electrochemical oxidation of the benzidine station to generate the corresponding dication also restricts the shuttling process of the bead, limiting it to the biphenol region of the dumb-bell (Fig. 1B). This conclusion is evidenced by comparison of the anodic voltammetric behaviour of **1** $\cdot$ [PF₆]₄ and two structurally related compounds (Fig. 4). The cyclic voltammetry of a poly-ether derivatized benzidine species (compound **2**, see structure in Fig. 4) and a simple benzidine rotaxane (**3** $\cdot$ [PF₆]₄, where **3** is shown in Fig. 4) have been described previously¹². In this rotaxane the bead cannot move too far from the benzidine donor station because it is confined in a shorter dumb-bell component. The half-wave potentials corresponding to the two consecutive one-electron oxidations of the benzidine subunit in **3** $\cdot$ [PF₆]₄ are thus substantially more positive than those observed in the uncomplexed, benzidine-containing thread (compound **2**). These potential shifts are a consequence of the unfavourable electrostatic repulsions between the tetracationic bead and the benzidine cation and dication residues that are electrogenerated in the anodic voltammetric scan. In the case of **1** $\cdot$ [PF₆]₄, only the half-wave potential for the first oxidation (benzidine to cation radical) is shifted in the positive direction as a result of the presence of the tetracationic bead. The second oxidation (cation

radical to dication) takes place at a half-wave potential which is essentially identical to that observed for the second oxidation in compound **2**. These results reveal that, in **1** $\cdot$ [PF₆]₄, the bead affects the first one-electron oxidation of the benzidine subunit as it shuttles along the whole length of the dumb-bell. But once a positive charge develops on the benzidine subunit, the bead does not affect significantly the second oxidation process because it is excluded from association with the benzidine station by coulombic repulsive forces.

The rotaxane described here exemplifies a class of molecular devices that has been termed controllable molecular shuttles^{24–26}. Other recent reports have described related pseudo-rotaxanes, rotaxanes and catenanes that can be controlled by photochemical^{27–29} or electrochemical³⁰ means. Unlike these, our system can be switched by two different mechanisms. Supramolecular chemists now are faced with two major challenges; first, to improve the molecular properties of these and related systems, and second, to find methods to 'wire' these novel molecules to the macroscopic world. □

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Trapping, detection and reaction of very large single molecular ions by mass spectrometry

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RECENT developments have made electrospray-ionization (ESI) mass spectrometry^{1–3} an important technique for measuring molecular weights and studying the structures of large molecules of relative molecular masses approaching 200,000 (M_r , 200K). Analysis of still larger molecules is difficult, however: to obtain a mass/charge ratio within the detectable limits, the ions must bear several charges, but a collection of ions of differing multiple charges presents a spectrum that is hard to interpret. This problem would be avoided if sufficient sensitivity can be achieved to detect individual molecules. Here we describe a technique that realizes this sensitivity for molecules of M_r to $\sim 7 \times 10^6$, and which has the potential to be extended to $M_r \approx 10^9$. We use ESI and Fourier-transform ion cyclotron resonance mass spectrometry⁴ to obtain repeated measurements of the mass-to-charge ratio of single, highly charged molecular ions. We are able to observe stepwise changes in the charge of an isolated ion owing to its reaction with small molecules introduced into the detection cell. This technique should have wide applications for characterization of polymers and large biomolecules.

The attributes of mass spectrometry include excellent sensitivity, broad applicability, and the capability for addressing complex mixtures and obtaining structural information for specific components. The electrospray ionization method involves the dispersion of liquids as highly charged micrometre-size droplets from which large molecules can be transferred to the gas phase^{1–3}. In comparison to other ionization techniques, ESI is gentler and generally results in extensive charging (z) which increases nearly linearly with relative molecular mass (M_r), facilitating analysis of large molecular ions within the mass-to-charge ratio (m/z) range of conventional mass spectrometers. The combination of ESI with mass spectrometry has provided M_r measurements with accuracies better than 0.01% (refs 2, 3), and has been shown effective for broad classes of synthetic polymers and biopolymers. Previous results have suggested that large molecules can be studied by the ESI process⁵, although doubts have been raised⁶. But because M_r determination and other applica-

tions for large molecules generally require the resolution of several different molecular-ion charge states, they become increasingly difficult as the molecular size increases.

In ESI-Fourier-transform ion cyclotron resonance (FTICR)⁷, ions are transported from the atmospheric-pressure ESI source, through regions pumped to obtain progressively lower pressures, and trapped under high vacuum in the box-shaped FTICR cell by a combination of static electric and magnetic fields. A radio frequency (r.f.) electric field applied at the cyclotron frequency excites ions to circular orbits between the two opposing detection plates, which make up two of the six cell walls⁸. The resulting oscillatory ion motion in the cell induces a transient signal in an external circuit at the ions' cyclotron frequency, allowing the simultaneous detection of ions across the m/z range^{4,8}. The resolution obtainable in such measurements is limited by the length of the transient signal, which is gradually damped by collisions and other factors⁹. Fourier transformation of this time-domain signal into the frequency domain provides a mass spectrum. Because detection is nondestructive, the same ions can be measured many times⁹. Our ESI-FTICR instrumentation incorporates a cryopump consisting of a cylinder cooled to ~ 15 K that extends into the bore of the 7-T superconducting magnet to the vicinity of the ICR cell, providing greatly improved pumping speeds and vacuum with ESI¹⁰. This allows the introduction of brief gas pulses to pressures of 10^{-4} to 10^{-3} torr and more effective trapping for large ions relative to earlier instruments.

An initial question was whether conventional broadband FTICR detection methods would have sufficient sensitivity for a single multiply charged ion. Previous estimates indicated detection limits in the range of 70–150 singly charged ions¹¹. In our studies, a poly(ethylene glycol) (PEG) of nominal M_r 5×10^6 yielded a mass spectrum consisting of hundreds of peaks from $m/z \sim 1,500$ –3,500. These peaks arise from single trapped ions having on average several thousand net positive charges per

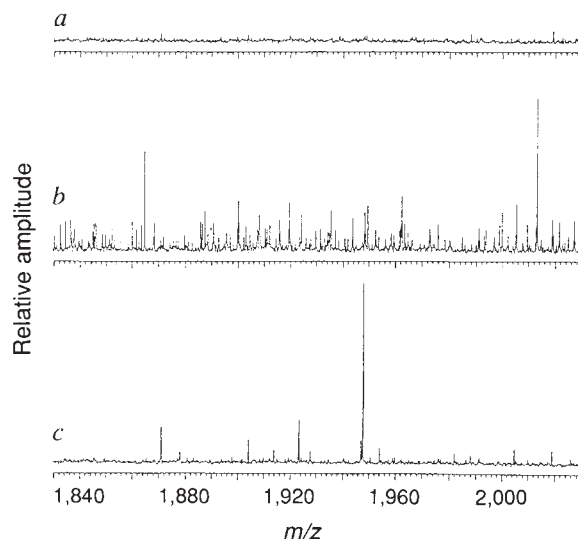


FIG. 1 Fourier-transform ion cyclotron resonance (FTICR) mass spectra for a small m/z region showing the background before injection of ions formed by ESI from a poly(ethylene glycol) (PEG) of nominal M_r 5×10^6 (a). Experiments were conducted in which ions were injected and accumulated in the cell for 1 s at 10^{-4} to 10^{-3} torr and collisionally cooled for 15 s, after which the trapping plate potentials were dropped from 3.5 to 0 V, allowing ions to diffuse out of the cell for periods of 0 (b) to 100 (c) ms before reinstating trapping potentials, and detection 5 s later. The slow diffusion rates of these ions compared to smaller ions (from ubiquitin (M_r 8.5K), albumin (M_r 66.5K), and a PEG of M_r 600K)) was qualitatively consistent with their size.

molecule due to Na^+ attachment (adduction)⁵. Figure 1 shows a small portion of a typical mass spectrum before (Fig. 1a) and after (Fig. 1b) ion injection. Each peak arises from a single ion, and the number of trapped ions may be decreased to any level desired by removing the voltages on the trapping plates and allowing ions to diffuse out of the cell (Fig. 1c). A number of experiments confirmed that these signals arose from single large ions, and not noise (or other detection artefacts) or from smaller

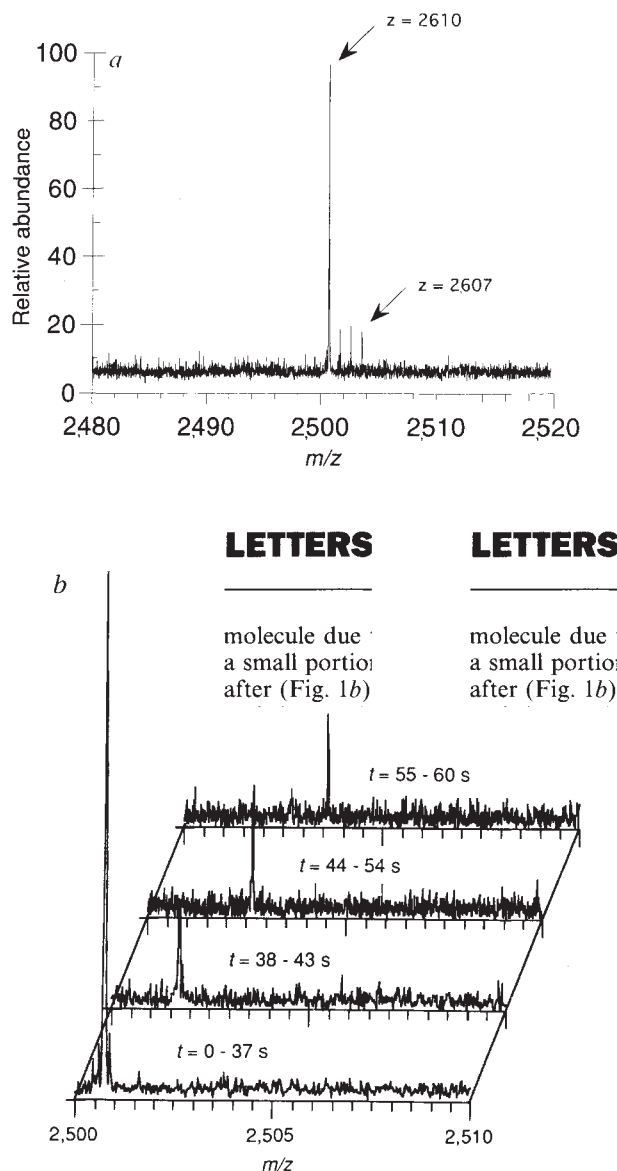


FIG. 2 Mass spectra illustrating peaks arising from different charge states (z) of 2,610 to 2,607 upon transformation of the full (a) and various regions (b) of a single 60-s transient obtained after isolating a single ion produced by electrospray ionization of a PEG sample of nominal M_r 5×10^6 . A single ion was first isolated by lowering the ion population, as demonstrated in Fig. 1, and using r.f.-excitation to eject all remaining ions from the cell except for the one desired⁸. The molecular ion was observed to shift in discrete steps, in m/z increments of ~ 0.95 , to higher m/z three times during the transient decay after the r.f.-excitation event. The m/z shifts are ascribed to charge state changes in single steps due to reactions. The different peak intensities derive from the different lifetimes (as well as radial amplitude) of each charge state. The assumption that these shifts arise from stepwise charge reduction allows an ion M_r of 6.527×10^6 to be determined.

ions. These experiments included: (1) measuring the time for diffusion of these large ions out of the cell compared to smaller protein ions (that is, the decrease in the ion population after dropping the trap plate potentials to 0 V for various periods), establishing that the ions were lost much more slowly than small proteins; (2) slowly changing the trapping potentials and observing the expected m/z shift, establishing that the peaks indeed arise from ions and not noise⁸; and (3) the observation of m/z shifts due to simple reactions, as discussed below. The peaks showed the signal levels, slow diffusion rates, and other behaviour expected for single ions having several thousand charges. Also noted was the absence of isotopic peaks, a characteristic of populations of smaller ions. Particularly supportive is the consistently quantized nature of the signals; that is, peak shifting, disappearance due to reaction, or diffusion out of the cell occurs in discrete steps, rather than by a continuous change in amplitude as would be observed for a population of smaller ions. A large ion can be stored in the cell for many hours, and identified repeatedly during this period.

The molecule's charge must be known to determine the M_r of large molecules by mass spectrometry, but existing methods are not applicable for a single ion. We are investigating two alternatives that involve (1) inducing a change in charge, and (2) inducing a change in mass by a simple adduction process. Both potentially provide mass measurement precisions of $<0.01\%$. In principle, a single reaction step provides sufficient information to determine M_r , but two or more reaction steps provide greater confidence.

As a demonstration of this approach we observed, over the course of numerous remeasurements, shifts to higher m/z for PEG ions reacting with added neutrals (crown ethers, to which we believe a sodium ion can be transferred) or residual background gas constituents. We also observed multiple reaction steps for a single ion during the transient decay from a single r.f.-excitation. Figure 2a shows a mass spectrum transformed from a 60-s transient decay after a single ion had been isolated. But by transforming only the appropriate portion of the transient, one can see that each species is observed at different times during the transient, as shown in Fig. 2b, and that the sudden disappearance of one species precisely coincides with the appearance of another. The stepwise m/z shifts shown in Fig. 2 allow the charge to be determined, yielding an ion M_r of 6.527×10^6 . We can thus observe the stepwise reactions of the ion, and in separate experiments have been able to observe the reactions of trapped ions for periods exceeding 10 hours. Single ions of the protein albumin ($M_r \sim 66\text{K}$) having as few as 30 charges have been detected with signal-to-noise levels of ~ 2 . The number of single ions that must be sampled to determine an average M_r depends on the heterogeneity, elemental composition and desired precision. Measurements for two PEG molecules of nominal M_r 5×10^6 , from an ideal M_r distribution arising only from the naturally occurring isotopic species, could yield an uncertainty of 0.01% (M_r 531), whereas 20 measurements afford an uncertainty of 0.001% , to 90% confidence. Determination of the average M_r obviously depends upon heterogeneity or polydispersity; generally large for synthetic polymers, but small for biopolymers. Such measurements will be substantially speeded by the ability to measure a number of single ions simultaneously by observing the time-resolved reaction steps in the course of a single transient, a technique we have recently developed and will report elsewhere¹².

Potential applications include the characterization of synthetic polymers, large structural proteins and DNA restriction fragments, for which M_r determinations should be at least 100 times more precise than by electrophoresis. DNA sequencing may be possible if a method can be developed to induce the stepwise degradation of a trapped oligonucleotide ion. It should also be possible to investigate selective non-covalent associations of small molecules with large molecules in solution¹³ by trapping such complexes and inducing their dissociation in the FTICR

cell. The upper M_r range is uncertain, but the initial electrospray droplet charging ($\sim 10^2$) and the m/z range available by FTICR ($>200,000$) suggest that molecules of M_r 10^9 may be amenable to study. $\square$

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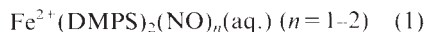
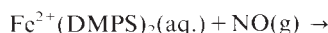
Removal of NO from flue gases by absorption to an iron(II) thiocholate complex and subsequent reduction to ammonia

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THE combustion of fossil fuels generates SO_2 and NO_x pollutants which cause acid rain and urban smog¹. Existing flue-gas desulfurization scrubbers involve wet limestone processes which are efficient for controlling SO_2 emissions but are incapable of removing water-insoluble nitric oxide. The current technique for post-combustion control of nitrogen oxide emissions, ammonia-based selective catalytic reduction, suffers from various problems^{2,3}, including poisoning of the catalysts by fly ash rich in arsenic or alkali, disposal of spent toxic catalysts and the effects of ammonia by-products on plant components downstream from the reactor. To circumvent the need for separate schemes to control SO_2 and NO_x , we have developed an iron(II) thiocholate complex that enhances the solubility of NO in aqueous solution by rapidly and efficiently absorbing NO to form iron nitrosyl complexes. The bound NO is then converted to ammonia by electrochemical reduction, regenerating the active iron(II) catalyst for continued NO capture. Our results suggest that this process can be readily integrated into existing wet limestone scrubbers for the simultaneous removal of SO_2 and NO_x .

We report here the NO absorption characteristics of a thiolated iron(II) chelate, $\text{Fe}^{2+}(\text{DMPS})_2$, where DMPS is 2,3-dimercapto-1-propanesulphonate ($\text{HSCH}_2\text{CH}(\text{SH})\text{CH}_2\text{SO}_3^-$).



Using a bubbling column (50 mm i.d., 420 mm long) in which simulated flue gas containing 300–600 p.p.m. NO is bubbled through a (red) solution of $\text{Fe}^{2+}(\text{DMPS})_2$ (10 mM), the NO absorption capacity^{4–7} of the resulting scrubber solution has

been measured with the aid of a chemiluminescent NO_x analyser. A typical NO absorption profile for a 10 mM $\text{Fe}^{2+}(\text{DMPS})_2$ solution under simulated flue gas scrubber conditions (580 p.p.m. NO, 55 °C and pH 6.6) is shown in Fig. 1a. By graphically integrating the absorption trace, the concentration of the NO adduct is obtained: for example, a 7.5 mM solution of $\text{Fe}(\text{DMPS})_2\text{NO}$ is produced in Fig. 1a. As flue gas contains 2–8% O_2 , Fig. 1b shows that the introduction of 5% O_2 reduces the NO absorption capacity of the solution by 44%. For comparison, a 10 mM $\text{Fe}^{2+}(\text{EDTA})$ solution under similar experimental conditions will produce the NO adduct at only 2.6 mM concentration, as shown in Fig. 1c. Introducing 5% O_2 into the flue gas stream results in a 83% reduction in the NO absorption capacity of the $\text{Fe}^{2+}(\text{EDTA})$ solution (Fig. 1d). It is clear that $\text{Fe}^{2+}(\text{DMPS})_2$ possesses a significantly larger NO absorption capacity than $\text{Fe}^{2+}(\text{EDTA})$ (2.5 times more anaerobically, and 7.5 times more in the presence of 5% O_2).

The visible spectrum of $\text{Fe}^{2+}(\text{DMPS})_2$ is characterized by a charge-transfer band at 508 nm. This band is no longer seen in the NO adduct, $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$. The infrared data (Fig. 2) show that $\text{Fe}^{2+}(\text{DMPS})_2$ binds a single NO molecule at low NO concentrations, and binds a second NO molecule at high NO concentrations. The presence of two types of nitrosyl complexes (Fig. 2) suggests that equation (1) can be rewritten in terms of two equilibria (K_1 and K_2) associated with the formation of nitrosyl complexes with: (1) only terminally-bound NO at low P_{NO} (<500 p.p.m.) and (2) terminal and bridging NO at higher P_{NO} (>500 p.p.m.), represented by the following reactions (2) and (3) respectively

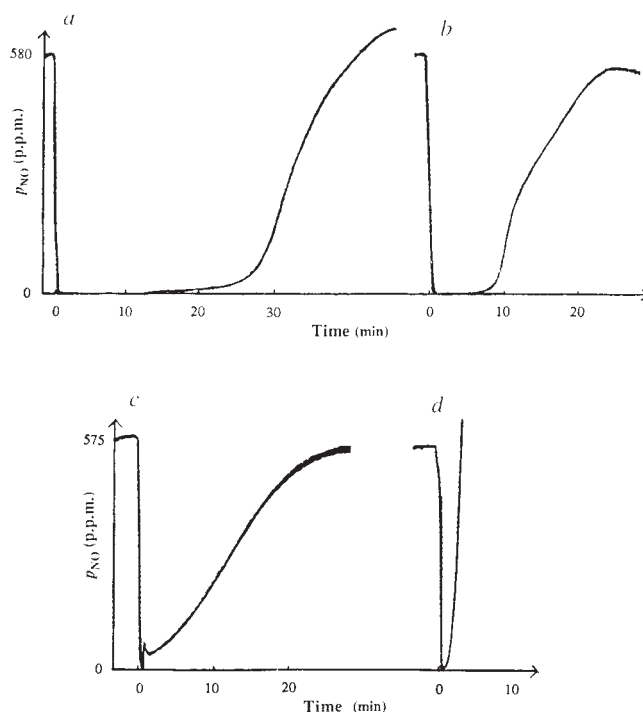
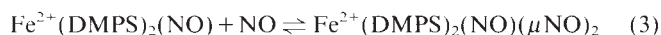
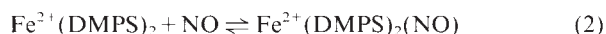


FIG. 1 NO absorption profiles (partial pressure of NO against time for: a, 10 mM $\text{Fe}^{2+}(\text{DMPS})_2$ with 580 p.p.m. NO and 0% O_2 (55 °C, pH 6) giving 7.5 mM $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$; b, 10 mM $\text{Fe}^{2+}(\text{DMPS})_2$ with 580 p.p.m. NO and 5% O_2 (55 °C, pH 6) giving 3.3 mM $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$; c, 10 mM $\text{Fe}^{2+}(\text{EDTA})$ with 575 p.p.m. NO and 0% O_2 (55 °C, pH 5.9) giving 2.6 mM $\text{Fe}^{2+}(\text{EDTA})\text{NO}$; d, 10 mM $\text{Fe}^{2+}(\text{EDTA})$ with 550 p.p.m. NO and 5% O_2 (55 °C, pH 5.8) giving 0.44 mM $\text{Fe}^{2+}(\text{EDTA})\text{NO}$).

where μNO stands for bridging NO. Both K_1 and K_2 have been determined from NO absorption experiments⁷ carried out at low and high NO (>500 p.p.m.) concentrations, respectively. At 55, 75 and 95 °C, the respective values of K_1 and K_2 (M^{-1}) are $2.1 \pm 0.2 \times 10^7$ and $6.9 \pm 0.1 \times 10^6$, $1.1 \pm 0.3 \times 10^7$ and $3.3 \pm 0.3 \times 10^6$, $6.9 \pm 0.2 \times 10^6$ and $1.8 \pm 0.2 \times 10^6$. For comparison, the equilibrium constant⁸⁻¹⁰ for the formation of $\text{Fe}^{2+}(\text{EDTA})(\text{NO})$ under typical flue-gas scrubber conditions, for example 300–600 p.p.m. NO, 55 °C, pH 6, is $1.0 \times 10^6 \text{ M}^{-1}$.

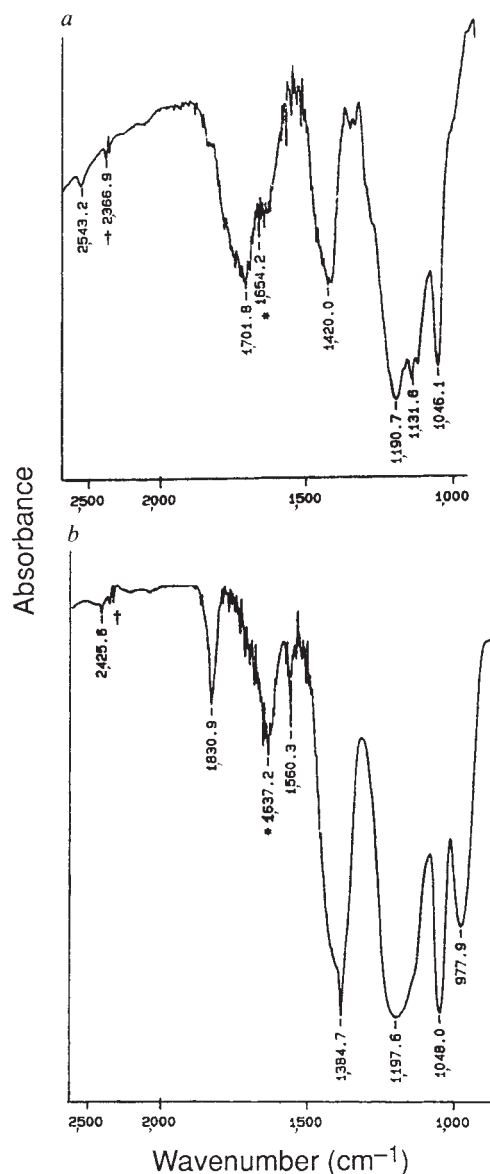
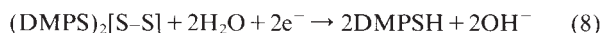
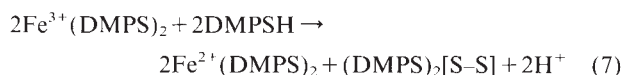
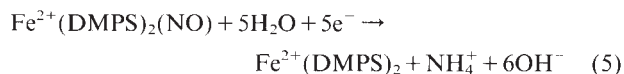
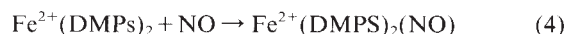


FIG. 2 Infrared spectra of a, $\text{Fe}^{2+}(\text{DMPS})_2$ with 300 p.p.m. NO (KBr pellet) main bands 2543 cm^{-1} (S–H), 1702 cm^{-1} (NO), 1420 cm^{-1} (C–H bend), 1191 and 1132 cm^{-1} (S=O); b, $\text{Fe}^{2+}(\text{DMPS})_2$ with 100% NO (KBr pellet), main bands 2426 cm^{-1} (S–H), 1830 cm^{-1} (NO), 1560 cm^{-1} (bridging NO), 1385 cm^{-1} (C–H bend), 1202 and 1137 cm^{-1} (S=O). (In both spectra, bands marked † and * are due to CO_2 and H_2O respectively.) The infrared spectra of $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$ show a characteristic absorption band for the terminally bound NO at 1702 cm^{-1} . But at increasingly higher NO concentrations, a new type of nitrosyl complex is formed featuring both terminal NO (1830 cm^{-1}) and bridging NO (1560 cm^{-1}). There is also a corresponding shift to lower frequency from 2543 cm^{-1} to 2426 cm^{-1} for the S–H stretching band in the new nitrosyl complex. The S–H bond in the new nitrosyl complex is thus weakened, indicating the participation of sulphur in bonding to the bridging NO, for example a $\text{Fe}-\text{N}(\text{=O})-\text{S}$ three-centred, two-electron bonding framework.

The higher value of the equilibrium constant for the formation of $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$, thus provides a good measure of the greater thermodynamic stability of the DMPS-nitrosyl complex, and largely explains the much more efficient absorption of NO by $\text{Fe}^{2+}(\text{DMPS})_2$ compared with $\text{Fe}^{2+}(\text{EDTA})$. The enthalpy and entropy for the formation of both types of nitrosyl complexes have been derived. These are $\Delta H_1^0 = -6.9 \pm 0.3 \text{ kcal mol}^{-1}$, $\Delta H_2^0 = -9.8 \pm 0.3 \text{ kcal mol}^{-1}$, and $\Delta S_1^0 = 8.2 \pm 0.4 \text{ e.u.}$, $\Delta S_2^0 = 5.5 \pm 0.4 \text{ e.u.}$ (e.u., entropy unit).

The presence of bisulphite/sulphite ions in solution did not alter the NO absorption characteristics, suggesting that SO_2 has no effect on the NO absorption capacity of $\text{Fe}^{2+}(\text{DMPS})_2$. Unlike $\text{Fe}^{2+}(\text{EDTA})(\text{NO})$, $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$ does not react with bisulphite/sulphite ions to yield undesired nitrogen-sulphur by-products^{11,12}.

The clean removal of the bound NO from $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$, to regenerate $\text{Fe}^{2+}(\text{DMPS})_2$ for sustained NO absorption, constitutes a critical step in the overall effectiveness of the metal chelate process for the removal of NO from flue gas. Moreover, the oxidation of the SH moiety in DMPS to a disulphide (S–S) linkage must be addressed as the thiol (S–H) group is needed to reduce Fe^{3+} to Fe^{2+} for sustained NO absorption. The electroreduction of a S–S linkage into a S–H bond has been achieved in the transformation of cystine to cysteine^{13,14}. In our work, the electrolysis has proved to be well suited to accomplish the dual task involved in the regeneration of $\text{Fe}^{2+}(\text{DMPS})_2$; (1) electrochemical removal of the bound NO, and (2) electroreduction of the S–S to the S–H moiety. The following scheme illustrates the chemistry involved:



Cyclic voltammetry of $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$ shows a reversible iron(II/III) redox couple and more significantly, a reduction wave due to NO reduction which can be seen at -0.75 V vs SCE (Fig. 3). During controlled-potential electrolysis experiments on the preparative scale, the products from the electroreduction of coordinated NO were collected in a H_2SO_4 trap (100 ml of $\sim 0.9 \text{ M H}_2\text{SO}_4$) and subsequently analysed by ion chromato-

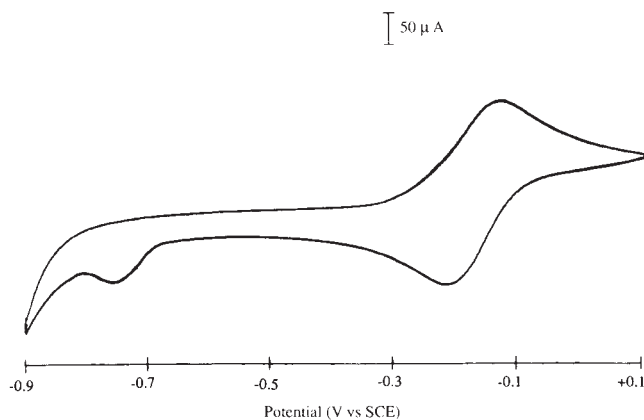


FIG. 3 Cyclic voltammogram of $\text{Fe}^{2+}(\text{DMPS})_2(\text{NO})$ at 25 °C and pH 6.0 (experimental conditions: $0.1 \text{ M Na}_2\text{SO}_4$ supporting electrolyte; $[\text{Fe}^{2+}(\text{DMPS})_2] = 10 \text{ mM}$; glassy carbon working electrode; Pt wire counter electrode; SCE reference electrode; scan rate 50 mV s^{-1}).

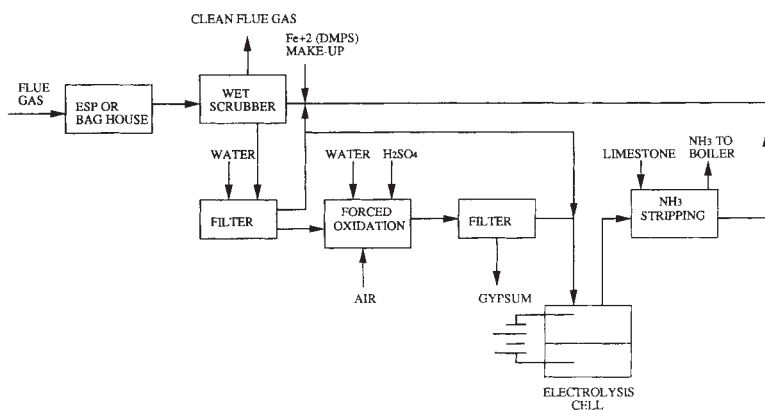


FIG. 4 Conceptual flow diagram of a metal-thiochelatate-based process for flue-gas clean-up. The coordinated NO is electrically reduced to produce NH_4^+ , which may be separated from the liquors by heating. The NH_3 produced may be injected into the boiler to reduce the concentration of NO_x based on the principle of selective noncatalytic reduction (SNCR).

graphy. Results consistently showed the formation of NH_4^+ in quantitative yield. This electrolytic approach leads to the regeneration of $\text{Fe}^{2+}(\text{DMPS})_2$ with no attenuation in its NO removal capacity, as monitored by visible spectroscopy and NO absorption experiments.

The commercial viability of this metal-thiochelatate-based process has been considered and the following practical and economic analyses suggest that the process represents a strong case for further industrial scale-up.

The NO absorption rate in metal chelate solutions has been found to be a liquid-film mass-transfer control process^{9,15,16}. Effective gas-liquid contact is vital to achieve 80% NO removal, which is the target for NO removal processes. A Thiosorbic lime pilot plant test¹⁶ with 10–15 mM $\text{Fe}^{2+}(\text{EDTA})$ showed that NO removal was ~30% at a flue gas velocity of 2.5 m s^{-1} and a L/G of 4.4 l m^{-3} (L/G is the ratio of the flow rate of scrubbing liquors (in l min^{-1}) to the flow rate of flue gas (in $\text{m}^3 \text{ min}^{-1}$)). The rate equations suggest that the absorption efficiency is improved^{9,15} with an increase in the equilibrium constants for NO absorption. Moreover, the absorption efficiency as transfer units¹⁶ increases in direct proportion both to the gas-liquid interfacial area and to absorber height. With the increase of L/G, installation of sieve trays, and/or use of a turbulent contact absorber¹⁷ to increase the interfacial area by a factor of ~4.5, the absorption rate of NO should reach 80%. The NO absorption rate of 80% efficiency by $\text{Fe}^{2+}(\text{EDTA})$ has been demonstrated¹⁸ at the pilot-plant stage with an L/G of $\sim 14 \text{ l m}^{-3}$. Preliminary bench-scale experiments indicated that the reaction rate of NO with $\text{Fe}^{2+}(\text{DMPS})_2$ was comparable to that with $\text{Fe}^{2+}(\text{EDTA})$. Pilot-plant tests are needed to determine the NO removal efficiency by $\text{Fe}^{2+}(\text{DMPS})_2$ under realistic operating conditions.

In a preliminary economic evaluation of material costs, a fair estimate of \$1.00 per lb DMPS is assumed. This estimate was derived¹⁹ based on the synthesis²⁰ of DMPS from allyl chloride, sodium sulphite, chlorine and sodium hydrosulphide. We have

considered reagent loss due to water entrainment in solid wastes. Under natural oxidation conditions where solid precipitates (filter cake of a mixture of calcium sulphite and sulphate) may occlude as much as 50% by weight of liquors, a wet limestone scrubber that removes 90% SO_2 from flue gas containing 2,000 p.p.m. SO_2 in a 500 MW coal-fired power plant would consume 2.7 short tons (ST; 1 ST = 2,000 lb) of DMPS per day due to entrainment, assuming the scrubbing liquors contain 10 mM $\text{Fe}^{2+}(\text{DMPS})_2$. If the addition of metal thiochelatate results in the removal of 80% NO from flue gas containing 375 p.p.m. NO (that is, 22.5 ST NO d^{-1}), then the DMPS loss is \$158 per ton NO_x removed. This loss may be reduced by washing the filter cake to recover the reagent. Under forced oxidation conditions where gypsum is precipitated, the reagent loss due to entrainment is ~\$31.6 per ton NO_x removed, because the gypsum precipitates occlude only 10% by weight of liquors. However, aeration under forced oxidation conditions would accelerate the oxidation of $\text{Fe}^{2+}(\text{DMPS})_2$. To get around this problem, the forced oxidation aeration may be performed after the extraction of entrainment liquors (Fig. 4).

As illustrated in equation (5), five electrons are needed to regenerate the spent scrubber solution. Whereas oxidation of the sorbent will actually require a total of nearly nine electrons to achieve the electroregeneration, we have found that addition of a five-fold molar excess of sodium thiosulphate will significantly slow down the oxidation of the chelate such that effectively, only six electrons are required for the regeneration. Based on the electricity cost of \$0.05 per kilowatt-hour, the electricity requirement for the process to absorb 300 p.p.m. NO and convert it to NH_3 suggests a cost of \$390 per ton NO_x removed. With nominal costs expected for additional equipment, this cost should compare favourably to the cost of \$2,000–4,000 per ton NO_x removed by the selective catalytic reduction process²¹. More realistic cost evaluations of this new process cannot be done until the completion of a pilot-plant test. □

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Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths

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In studies of moths flying upwind to a pheromone source, attention has focused on the influence on flight orientation of the composition^{1,2} and concentration^{3,4} of the chemical message, and of changes in the visual environment^{5,6} and in wind speeds⁷⁻⁹. The chemical signal must be intermittent for moths to fly upwind¹⁰⁻¹², when they usually follow a zigzag track, the evident expression of a self-steered counterturning programme^{13,14}. The integration of counterturning and optomotor anemotaxis allows insects to polarize the zigzags upwind in odour plumes^{10,15}. Not all moths, however, zigzag along a plume^{16,17}. It has been suggested that the propensity to zigzag or to fly straight upwind is related to the frequency at which males encounter pheromone filaments that comprise the plume, as well as the male's latency of response, characteristic for each moth species, to both the onset and loss of contact with filaments¹⁸. Here we present evidence that flight manoeuvres are dictated by the interactions of the male with individual odour pulses. We use *Cadra cautella*, the almond moth, to show how the structure of an odour plume^{19,20} can greatly modify the flight track. Males following either turbulent or mechanically pulsed plumes fly faster and straighter upwind, and locate sources more frequently than males following continuous narrow plumes. Males also fly straighter upwind to fast-pulsed plumes than to slow-pulsed plumes. The temporally modulated interplay between counterturning and optomotor anemotaxis that is induced by the plume's structure therefore seems to explain the manoeuvres and resultant flight track shapes made by *C. cautella* males when flying upwind towards a pheromone source.

Cadra cautella males were tested in a wind tunnel²¹ using either ribbon or turbulent pheromone plumes at two concentrations.

A ribbon plume consisted of a single continuous filament of odour. A turbulent plume was characterized as a stream of odour pulses separated by gaps of clean air (Table 1a). More males that had been exposed to turbulent plumes took off, locked onto the plume, and landed on the pheromone source, than males exposed to ribbon plumes (Table 1a). Males flying along ribbon plumes zigzagged slowly upwind, whereas males flying along turbulent plumes flew rapidly and often took nearly straight upwind courses irrespective of concentration²¹ (Fig. 1).

The tempo of a self-steered, counterturning programme in some moths is set by the instantaneous concentration of pheromone²². Although an increase in pheromone dose slightly decreased the tempo of self-steered turns in *C. cautella* males, the manipulation of the plume structure had strong effects on form of turns and interturn duration (Table 1a). In ribbon plumes, the frequency of turns across the wind line and lateral displacement were maximized: track angles were steered almost perpendicular to the wind line, which resulted in substantial lateral excursions (Fig. 1a, b). Males flying in turbulent plumes appeared to suppress counterturning, so that interturn duration and lateral displacement were minimized (Fig. 1b, c and Table 1a).

To isolate the effect of intermittency of turbulent plumes on male flight, plumes of defined pulse frequencies (Table 1b) were generated with a mechanical pulser^{21,23}. Continuous ribbon plumes resulted in slow zigzag flights (Fig. 2a). High-pulse frequency resulted in fast flights and straight tracks (Fig. 2c, d). The flights in low-pulse-frequency plumes alternated between a stationary zigzag during clean air intervals (casting) and short upwind surges, suggesting that males were responding to single pulse contacts²¹ (Fig. 2b). To determine whether the flight changes in response to an encounter with a single puff of pheromone, casting *C. cautella* males were exposed to a single, brief 0.25-s pulse (Fig. 3). The typical response of casting *C. cautella* males after a short delay was to turn upwind and engage briefly in a faster and straighter flight along the wind line (Fig. 3), a result found for another moth species²⁴.

Males thus change their flight in response to changes in the plume's overall shape (Fig. 1), and also to fine-scale variations in its internal structure (Figs 2 and 3). The characteristics of upwind flight in a plume may be predicted by a model based on the interactions of flying males and the individual pheromone pulses, assuming that straight flight results from the suppression

TABLE 1 Factors influencing male moth flight in odour plumes

Plume (a)	Pulse duration (s)	Air-gap duration (s)	Males landing (%)	Interturn duration (s)	Ground speed (cm s ⁻¹)	Track angle (deg)
Ribbon, low dose	Continuous		20 C	0.20 ± 0.02 C	36.17 ± 7.26 B	62.02 ± 7.96 A
Ribbon, high dose	Continuous		54 B	0.21 ± 0.04 C	35.19 ± 6.41 B	58.53 ± 9.37 A
Turbulent, low dose	0.17 ± 0.4	0.25 ± 0.04	69 A	0.30 ± 0.06 B	49.92 ± 9.62 A	41.46 ± 12.05 B
Turbulent, high dose	0.17 ± 0.04	0.25 ± 0.04	63 AB	0.36 ± 0.01 A	43.65 ± 8.34 B	34.30 ± 11.60 B
(b)						
Ribbon	9.67 ± 0.03	0.017 ± 0.03	83 B	0.25 ± 0.03 B	38.8 ± 7.24 B	63.5 ± 6.59 B
Slow pulse	0.17 ± 0.03	1.450 ± 0.04	63 C	0.29 ± 0.07 B	42.5 ± 4.25 B	73.9 ± 7.19 A
Fast pulse	0.13 ± 0.03	0.083 ± 0.01	100 A	0.40 ± 0.09 A	58.0 ± 11.05 A	40.4 ± 14.04 C

Values are means ± s.d. a, Males flying in turbulent plumes turn less frequently, fly faster, and give smaller track angles than males flying to ribbon plumes. A turn was scored when the track angle value changed signal relative to the wind direction from positive to negative or vice versa. Differences in pulse duration ($n=100$ pulses), air gap between pulses ($n=100$ gaps), percentage of males landing on the source, interturn duration ($n=20$ tracks), mean ground speed ($n=20$ tracks) and track angle ($n=20$ tracks) of flight are shown. Treatments were tested on a complete block randomized design. Values in the same column having none of the letters A-C in common are significantly different ($\alpha=0.05$, LSD, ANOVA, SAS). Methods as for Fig. 1. b, Males flying to quickly pulsed plumes turn less frequently, fly faster, and exhibit smaller track angles than males flying to ribbon plumes or to slowly pulsed plumes. These results illustrate the extremes of the plume structures tested in ref. 21. Flight tracks in plumes of intermediary pulse frequency showed intermediate values for the flight parameters measured. Methods as for Fig. 2.

FIG. 1 Example of *Cadra cautella* male flight tracks in ribbon plumes and turbulent plumes at two concentrations. Data points represent the position of the moth at 0.03-s intervals. Males flying to ribbon plumes produce zig-zagging flight tracks (a and b), and males flying to turbulent plumes produce straight tracks (c and d), independent of concentration. Frequency distribution histograms of pooled track angles (insets) are bimodal, with angles clustered around -90° and 90° , or unimodal with a mode of 0° (0° is upwind, 90° crosswind and 180° downwind). Bimodal distribution reflects crosswind tracks typical of flights in ribbon plumes (a and b). A mode of 0° reflects straight, upwind flight or a track with reversal legs nearly straight upwind as observed in turbulent plumes (c and d). METHODS. Pheromone sources (0.7-cm-diameter filter paper) containing 0.45 ng (low dose) or 45 ng (high dose) of pheromone were placed in 45 cm s^{-1} winds to generate continuous ribbon plumes of $0.8 \pm 0.2 \text{ cm}$ width and $0.1 \pm 0.1 \text{ cm}$ height. Addition of a $3 \times 3 \text{ cm}$ wind deflector standing 4 cm upwind created turbulent plumes. Males were released 1 m downwind from the pheromone source. Flight tracks of 20 males for each combination of pheromone dose and plume structure were videotaped and the video images analysed frame-by-frame as described in ref. 25. Statistical analysis as in ref. 21.

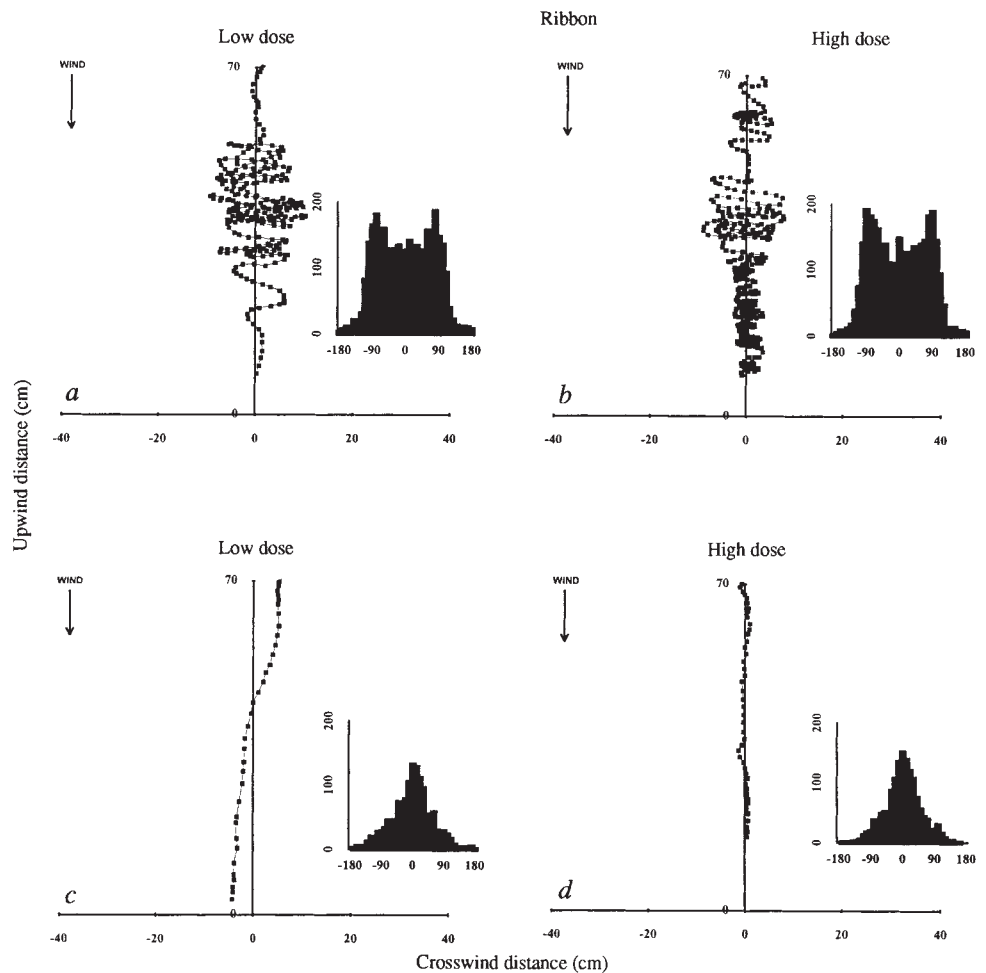


FIG. 2 a, The effect of manipulating the structure of pheromone plumes on the flight track of male moths by pulsing pheromone. Representative flight tracks and the corresponding distribution of flight-track angles of all males in each treatment are shown. Males flying to ribbon (a) and to slowly pulsed plumes (b) produced zigzagging flight tracks; and males flying to more quickly pulsed plumes (c and d) produced essentially straight tracks. Male lateral displacement in ribbon and slowly pulsed plumes is elaborated as frequent, conspicuous zigzags (a and b). Most flight tracks in quickly pulsed plumes lack the same temporal organization of lateral displacement (c), but some flight tracks have vestiges of counterturning (d). METHODS. Plumes were generated by forcing air regulated by a flow controller apparatus (SFC-2, Syntech)^{21,23} through a glass chamber containing a filter paper with 4.5 ng pheromone and injecting the exhaust in the centre of the working area of the wind tunnel with winds of 45 cm s^{-1} . The flight tracks of 20 males along structurally different plumes were videotaped and the resulting images analysed^{21,25}.

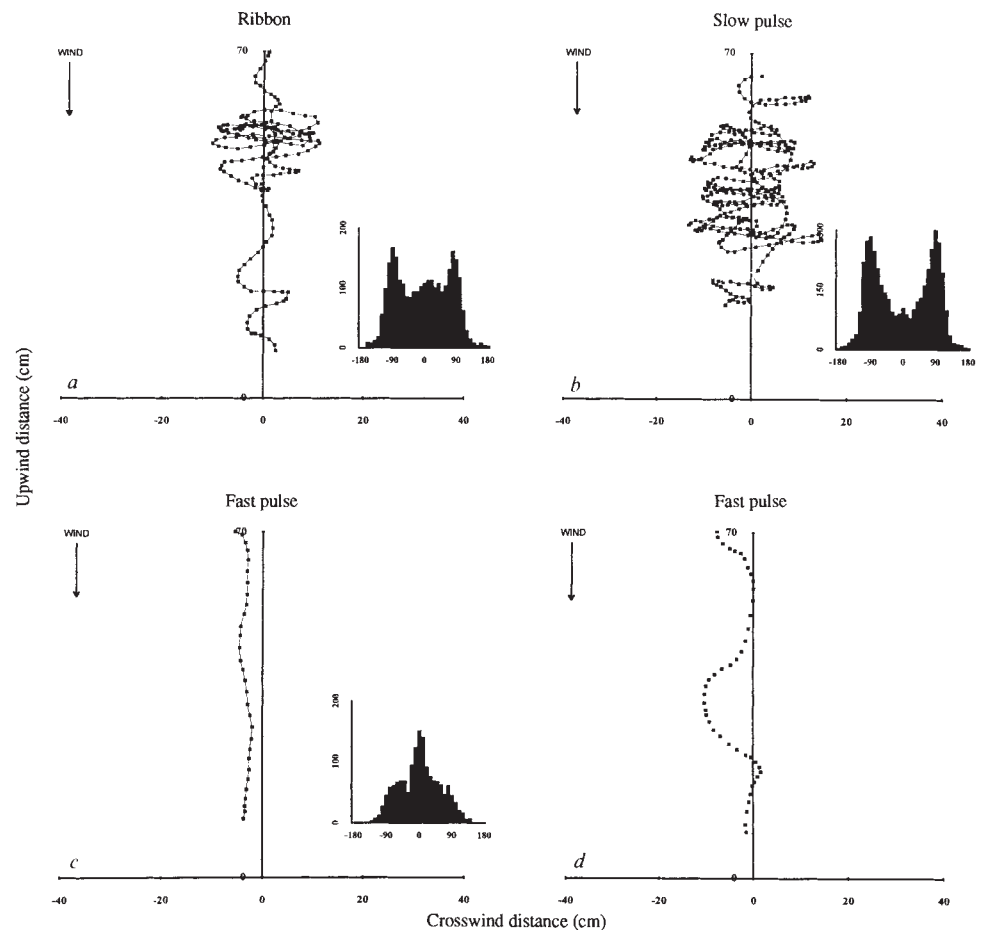
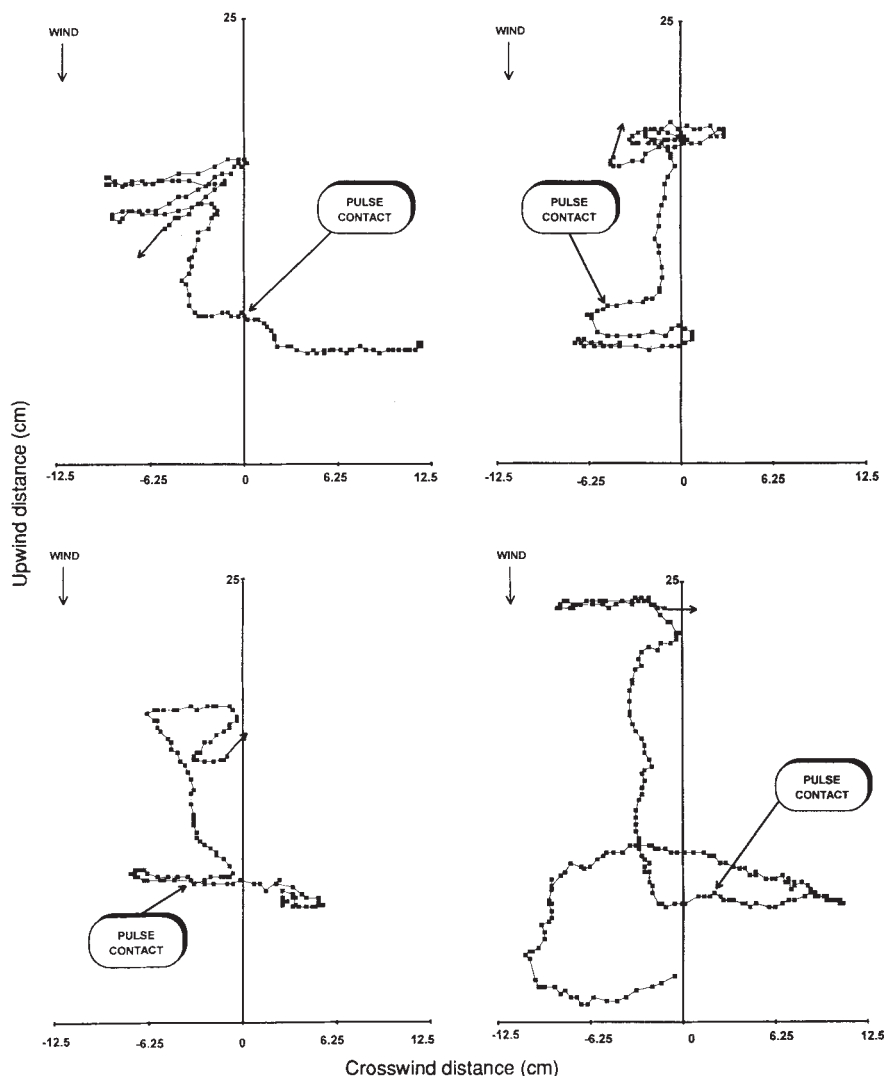


FIG. 3 Examples of the characteristic change of flight behaviour of *C. cautella* males in response to contact with a single pheromone pulse. Males flying upwind in a fast-pulsed pheromone plume were induced to cast as a result of removal of the plume. Once casting in clean air the male was exposed to a single pheromone pulse. Contact with pheromone (arrow) was followed by a sharp turn upwind, and a faster and straighter upwind flight. In the absence of new pheromone pulses, males returned to casting flight.

METHODS. Fast-pulsed plumes and single pulses were generated by forcing air regulated by the flow controller apparatus through a glass chamber containing a filter paper with 4.5 ng pheromone and injecting the exhaust in the centre of the working area of the wind tunnel with winds of 50 cm s^{-1} . Frame-by-frame video analysis (60 frames s^{-1}) of single TiCl_4 'smoke' pulses generated under the same experimental conditions as pheromone pulses allowed accurate prediction of the pheromone pulse position in the working area with time. An audio and a visual signal flagged the generation of single pulses. Flight tracks were analysed for males that intercepted the pheromone pulse. Moths intercepted pulses of $7.5 \pm 1.25 \text{ cm}$ cross-section along the wind line. Data points represent the position of the moth at 0.017-s intervals.



of a moth's internal counterturning programme¹⁸. For straight upwind flight to occur to turbulent plumes, the following elements are required: (1) a tempo of pheromone pulses above a certain frequency, so the male antennae encounter a pulse before the male starts to countturn¹⁸; (2) a refractory period during which contact with pheromone or clean air has no effect on behaviour²¹; and (3) a level of pheromone concentration in the pulse above threshold for upwind flight behaviour, but below levels that promote turns more perpendicular to the wind line²¹. Pulses should be sufficiently brief along the wind line to promote brief encounters with the antennae and 'disappear' to then be replaced by clean air (or by low concentration) before the male starts counterturning. Crosswind diameter of pulses should be large enough that contact with a new pheromone pulse occurs before the end of the period in (1), even if the male's flight

track deviates slightly from the central axis of the plume. The integration of counterturning and optomotor anemotaxis modulated by the structure of an odour plume seems to explain many features of the moth's flight manoeuvres along plumes of differing form.

The fact that frequently straight flights were seen in moths flying along large turbulent plumes and high-frequency pulsed plumes, and rarely in moths flying along small, continuous plumes or low-frequency pulsed plumes²¹, supports this model. We conclude that the interaction of the moth with the structure of the plume is an integral part of the flight guidance system, modifying the output of the internal turn-generator and the optomotor anemotactic mechanism. Future studies of insect orientation to odour should account for the pronounced effects of plume structure. □

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Male mutation rates and the cost of sex for females

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ALTHOUGH we do not know why sex evolved, the twofold cost of meiosis for females provides a standard against which postulated benefits of sex can be evaluated¹. The most reliable benefit is sex's ability to reduce the impact of deleterious mutations^{2,3}. But deleterious mutations may themselves generate a large and previously overlooked female-specific cost of sex. DNA sequence comparisons have confirmed Haldane's suggestion that most mutations arise in the male germ line^{4,5}; recent estimates of α , the ratio of male to female mutation rates, are ten, six and two in humans, primates and rodents, respectively⁶⁻⁸. Consequently, male gametes may give progeny more mutations than the associated sexual recombination eliminates. Here I describe computer simulations showing that the cost of male mutations can easily exceed the benefits of recombination, causing females to produce fitter progeny by parthenogenesis than by mating. The persistence of sexual reproduction by females thus becomes even more problematic.

Figure 1 shows three representative selection functions⁹, and Fig. 2 some results of simulations using them. All parthenogenetic populations (heavy lines in Fig. 2) reach mutation/selection equilibria at a mean fitness, W_{eq} , of $e^{-\mu_f}$, where μ_f is the genomic mutation rate in females^{10,11}. Under independent selection (Fig. 2a), when the male genomic mutation rate $\mu_m = \mu_f$ sexual reproduction does not change the population's fitness because, with no epistasis, mutations remain randomly distributed and recombination can have no effect^{11,12}. If $\mu_m > \mu_f$, sex drastically reduces fitness; when $\alpha = 2, 6$ and 10 , W_{eq} falls from 0.74 to 0.64, 0.35 and 0.19, respectively, precisely the fitnesses predicted by the new mean mutation rates of 0.45, 1.05 and 1.65.

Selection functions incorporating epistasis let us evaluate sex when both recombination and male mutations are affecting mutation load. The moderate epistasis of the quadratic function (Fig. 1b) reproduces the observed effects of spontaneous muta-

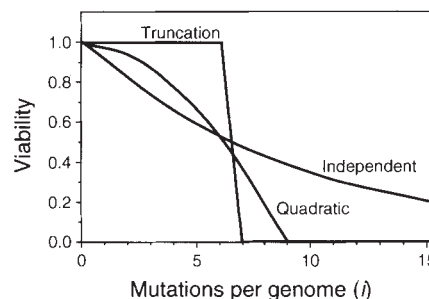


FIG. 1 Selection functions used in the model. i , Number of mutations per genome; v , viability. Independent selection: $v = 0.9^i$. Quadratic selection: $v = 1 - 0.014i - 0.0112i^2$ (ref. 9). Truncation selection: $v = 1$ for $i < 7$, $v = 0$ for $i \geq 7$.

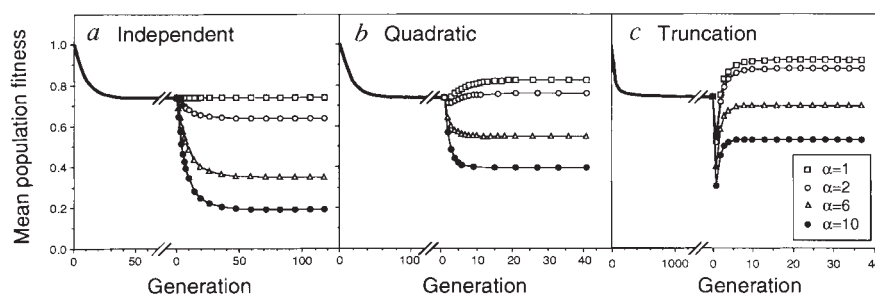
tions in *Drosophila*^{9,13}. Here sexual reproduction increases fitness both when $\alpha = 1$ (to 0.83) and when $\alpha = 2$ (to 0.76), but reduces it if $\alpha = 6$ or 10 (to 0.55 or 0.40). Under truncation selection, accumulating mutations do not change viability until a threshold is reached (here 7) where it falls to zero. This extreme epistasis allows recombination to increase fitness greatly; when $\alpha = 1$ sex raises W_{eq} to 0.96 (Fig. 2c). Even here elevated male mutation rates greatly diminish the benefits of sex, eliminating them whenever $\alpha > 5$. (Fitness falls transiently in the first sexual generation because reassortment of the mutations accumulated under parthenogenesis suddenly exposes them to selection.)

Figure 3 shows the reverse situation for a wide range of mean mutation rates (quadratic selection, $\alpha = 6$). Each line shows first the equilibrium fitness of a sexual population with a particular mean μ , followed by the change in fitness after parthenogenesis is adopted. The mutation load ($1 - W_{eq}$) is more than halved by parthenogenesis at low mutation rates ($\mu \leq 0.45$), and fitness itself is more than doubled at high rates ($\mu \geq 2.6$). Under parthenogenesis the fitness initially overshoots W_{eq} because of the favourable mutation distribution present at the sexual equilibrium. This also occurs under truncation selection (not shown), and may be evolutionarily significant, as it gives parthenogenesis a short-term benefit even when the equilibrium fitness favours sex.

Because we cannot generalize about the selective consequences of the 'average' mutations assumed by selection functions, I have

FIG. 2 Mean fitnesses of parthenogenetic and sexual populations under different selection functions and mutation rates. Heavy lines, parthenogenetic populations ($\mu_f = 0.3$) approaching equilibrium. Light lines, sexual populations derived from the asexual populations. a, Independent selection; b, quadratic selection; c, truncation selection. Squares, $\alpha = 1$; empty circles, $\alpha = 2$; triangles, $\alpha = 6$; filled circles, $\alpha = 10$.

METHODS. The model compares parthenogenesis and sexual reproduction by deterministically simulating large populations undergoing repeated generations of viability selection (and reproduction), deleterious germ-line mutation and, where appropriate, sexual recombination by meiosis and gamete fusion. Populations are characterized by their distribution of mutations and by the associated mean fitness W (the fraction of the population that will survive the next round of selection against these mutations). Each simulation is continued until the population reaches an equilibrium where mutagenesis is exactly balanced by selection. The simulation is written in ThinkPascal, and run on a Macintosh LCIII. Populations are described by their mutation distributions. The relative frequencies of individuals with different numbers of mutations after selection, male and female germ-line mutagenesis, and recombination are given by the mutation distributions $\mathbf{S}$, $\mathbf{M}_m$ or $\mathbf{M}_f$, and $\mathbf{R}$. The i th term of each distribution has the frequency of individuals with i mutations (i ranges from 0 to i_{max}). The value of i_{max} was varied between 15 and 50 to minimize runtime



while keeping matrix truncation errors below 10^{-7} . All mutations are unlinked and equally deleterious. Selection: Mutations are co-dominant. Selection functions are described in Fig. 1. Reproduction occurs by normalization of $\mathbf{S}$. Mutagenesis: The probability that the male or female acquires i new mutations is a Poisson distribution with mean μ_m or μ_f . Recombination: Mutation distributions in male and female gametes ($\mathbf{G}_m$ and $\mathbf{G}_f$) are calculated from $\mathbf{M}_m$ and $\mathbf{M}_f$. The progeny distribution $\mathbf{R}$ is then the product of the gamete distributions. Mating is thus random and there is no linkage. Validation of the model: The model was shown to give the expected results under conditions where W_{eq} is known from analytical results: (1) under parthenogenesis: $W_{eq} = e^{-\mu_f}$; (2) under independent selection: $W_{eq} = e^{-\mu}$, where $\mu = \mu_m(1 + \alpha)/2$; (3) using the selection function described by Charlesworth ($v = e^{-ai - bi^2}$), for which W_{eq} has been determined by analytical approximation³.

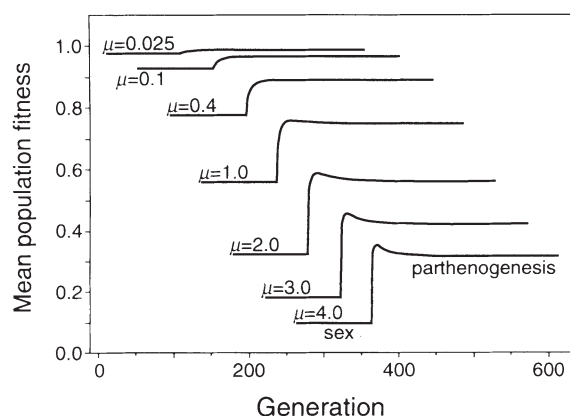
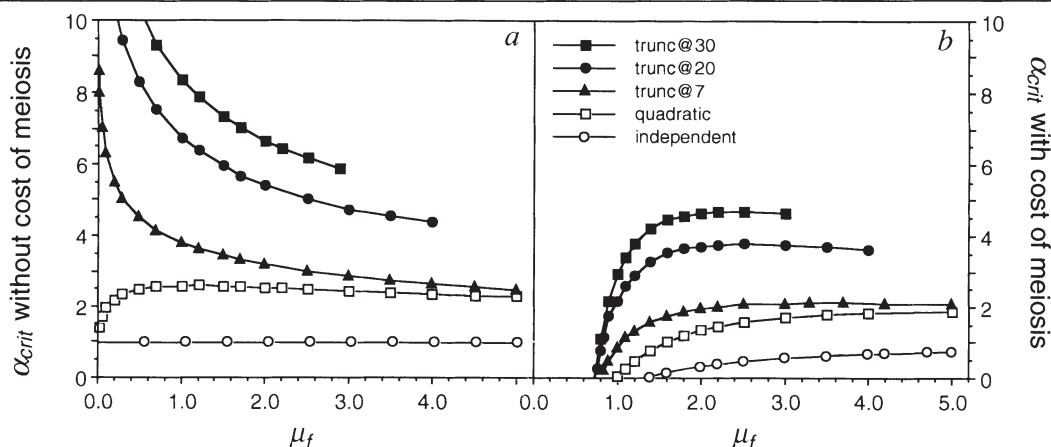


FIG. 3 Mean fitnesses of populations switching from sexual reproduction to parthenogenesis. Mean mutation rate is indicated to the left of each line. Selection is quadratic and $\alpha = 6$. Each population begins at its sexual equilibrium. For clarity, lines are shown staggered by 40 generations.

FIG. 4 Critical values of α , at which sexual and parthenogenetic reproduction give the same equilibrium population fitness. Selection functions: empty circles, independent; empty squares, quadratic; filled triangles, truncation at 7; filled circles, truncation at 20; filled squares, truncation at 30. *a*, Fitnesses neglect the twofold cost of meiosis for females. *b*, Fitnesses corrected for the twofold cost of meiosis.



examined fitness under a range of conditions. The truncation and independent functions were chosen to represent absolute and no epistasis, respectively, with selection intensities equivalent to that of the biologically based quadratic function. Because most real mutations probably have very minor effects on fitness, I have also tested truncation functions with thresholds of 20 and 30. Figure 4a summarizes the results for all these functions by comparing, for different mutation rates, values of α at which parthenogenesis and sex give the same equilibrium fitness (α_{crit}). Under independent selection $\alpha_{crit} = 1$. With quadratic selection, parthenogenesis is favoured when $\alpha > 2.5$, unless the mutation rate is very low. Truncation selection gives similar α_{crit} at high mutation rates, but at low rates sexual females can tolerate higher values of α .

In many sexual species females do all of the work of reproduction but can pass on only half of their genes. The analysis in Fig. 4b incorporates this cost by requiring that each sexual population's fitness be twice that of the corresponding parthenogenetic population. This precludes any net advantage of sex if $\mu_f < 0.7$, or under independent selection (unless $\alpha < 1$). Increasing the epistatic component of selection increases the benefits of recombination and thus the tolerance of male mutations, but under the conditions examined α_{crit} never exceeds 5 and shows little dependence on mutation rates above $\mu_f = 2$. However, α_{crit} may be larger under selection functions, allowing still more mutations to accumulate. Stochastic effects, known to increase the costs of deleterious mutations¹⁴, could also increase (or decrease) tolerance of elevated male mutation rates.

Elevated germ-line mutation rates may be an inevitable consequence of producing large numbers of gametes, male or female. Chang *et al.*⁸ found a correspondence between male and female point mutation rates and the number of cell divisions in the corresponding germ lines, supporting the hypothesis that these mutations arise during DNA replication. This predicts that α will be highest in species where males produce far more gametes than do females, for example, wind-pollinated plants and aquatic animals with external fertilization.

Given that even small increases in mutation rates significantly decrease fitness, how might parents reduce the number of new mutations in their progeny? The obvious solution, increasing the fidelity of germ-line-specific DNA polymerases and DNA repair pathways, will slow cell division and may decrease male fecundity. Females with physiological commitments preventing a switch to parthenogenesis (such as mammals) might reduce the contribution from male mutations by mating with the youngest males available, as their gametes will have undergone the fewest rounds of DNA replication. Although each generation's new mutations make only a minor contribution to total mutation load, the cumulative effect of reducing them can be substantial.

Kondrashov has shown that in large populations recombination of deleterious mutations can overcome the twofold cost of sex for females only if mutation rates are high ($\mu_f > 1$) and selection incorporates substantial epistasis^{2,15,16}. Unfortunately, the simulations described above suggest that, even when these conditions are met, females will be selected to give up sex entirely if male mutation rates are excessively high. That they have not done so leaves us in a quandary. If male mutations are not the major cost to females that this model suggests, it should be because deleterious mutations are less important than we have thought. But what could then pay the twofold cost of meiosis for females? One possibility is the antagonistic selection imposed by coevolving parasites, which, in combination with selection against deleterious mutations, can greatly increase the benefits of sex^{17,18}. To clarify these issues we urgently need more data about the rates and selective consequences of real mutations in real populations. □

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Antigen processing and class II MHC peptide-loading compartments in human B-lymphoblastoid cells

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THE peptide/class II major histocompatibility complex (MHC) complexes recognized by CD4⁺ T cells have been characterized at the structural¹ and biochemical^{2–4} levels and studies on the transport and maturation of class II MHC indicate that specialized sites may be involved in peptide acquisition^{5–11}. Here we report the characterization of the compartments involved in antigen processing and class II MHC loading relative to distinct functional domains of the endocytic pathway in antigen-specific human B lymphocytes^{12–14}. Peroxidase-mediated crosslinking analysis¹⁵ in intact cells demonstrates that peptide loading of class II MHC takes place in a compartment accessible to membrane immunoglobulin but not to transferrin receptors, although processing may be initiated within the latter domain. The density of membrane vesicles carrying newly assembled class II MHC complexes was distinct from early and late endosomes and dense lysosomes. Endocytosed antigen–gold complexes enter a class II MHC-rich compartment morphologically very similar to that described previously⁹ and within the time frame of biochemically detectable peptide loading.

Tetanus-toxin-specific human B-lymphoblastoid cells were pre-loaded with ¹²⁵I-labelled antigen at 0 °C, washed and incubated at 37 °C^{13,14}. After 30 min, sodium dodecyl sulphate (SDS)-stable class II $\alpha\beta$ dimers become labelled with processed tetanus toxin peptides (ref. 16 and Fig. 1a). In this system, surface expression of antigen-loaded class II MHC, detected either by T-cell assay¹⁷ or by direct surface biotinylation (S. Moore and C.W., unpublished results), occurs 45–60 min after antigen uptake, that is ~15–30 min after their intracellular assembly. To analyse the intracellular location of newly assembled complexes, we first used a non-disruptive method of cellular compartmental analysis. Endocytosed horseradish peroxidase (HRP) or HRP-conjugates generate a dense crosslinked polymer on incubation with 3,3'-diaminobenzidine (DAB) and H₂O₂. Only proteins within the compartments carrying the HRP probe are polymerized and rendered detergent-insoluble^{15,18}. Thus, different peroxi-

dase conjugates (such as transferrin–HRP (Tf–HRP) or antigen–HRP) can be used to probe the location, in intact cells, of distinct biochemical intermediates in antigen processing. Control experiments demonstrated very efficient H₂O₂-dependent polymerization (>90%) of the Tf–HRP probe and co-internalized transferrin, which fail to enter the stacking gel (Fig. 1b). To analyse the location of newly assembled intracellular peptide/class II MHC complexes, antigen-specific B-lymphoblastoid cells were pre-loaded with ¹²⁵I-labelled antigen at 0 °C and chased at 37 °C to allow antigen uptake and processing. Tf–HRP was included during the last 30 min. Cell aliquots were incubated with DAB at 0 °C in the presence or absence of H₂O₂, then class II MHC molecules were immunoprecipitated and analysed by SDS-PAGE. Class II $\alpha\beta$ dimers, labelled with processed antigen-derived peptides after 30 or 60 min, escaped crosslinking by Tf–HRP (Fig. 1c, lanes 1 and 2, 3 and 4). In contrast, when antigen–HRP replaced Tf–HRP during the chase, intracellular class II complexes were now efficiently crosslinked and therefore not immunoprecipitated (>60%; Fig. 1c, compare lanes 7 and 8). (A cohort of unoccupied membrane immunoglobulin recycles to the cell surface under these conditions¹⁴ allowing virtually simultaneous uptake of both radiolabelled and peroxidase-conjugated antigen.) Mixing experiments established that crosslinking of class II $\alpha\beta$ dimers was occurring within intact cells (lanes 9 and 10; see legend) and was not simply due to greater uptake of this conjugate compared to the Tf–HRP conjugate (not shown).

In the same cells we also assessed the distribution of a set of non-MHC-associated clonotypic antigen fragments (*M_r* range 3K–16K) that remain bound to immunoglobulin¹³. In contrast to peptide-loaded class II MHC molecules, a significant proportion (40–60%) of these antigen fragments were consistently crosslinked by Tf–HRP (Fig. 1c, lanes 13 and 14). Control experiments established that this was mediated by specific uptake of Tf–HRP and was not due to misrouting of the Tf–HRP conjugate (see legend). When the antigen–HRP probe was used, almost complete (80–90%) crosslinking of these fragments occurred (Fig. 1c lanes 15 and 16).

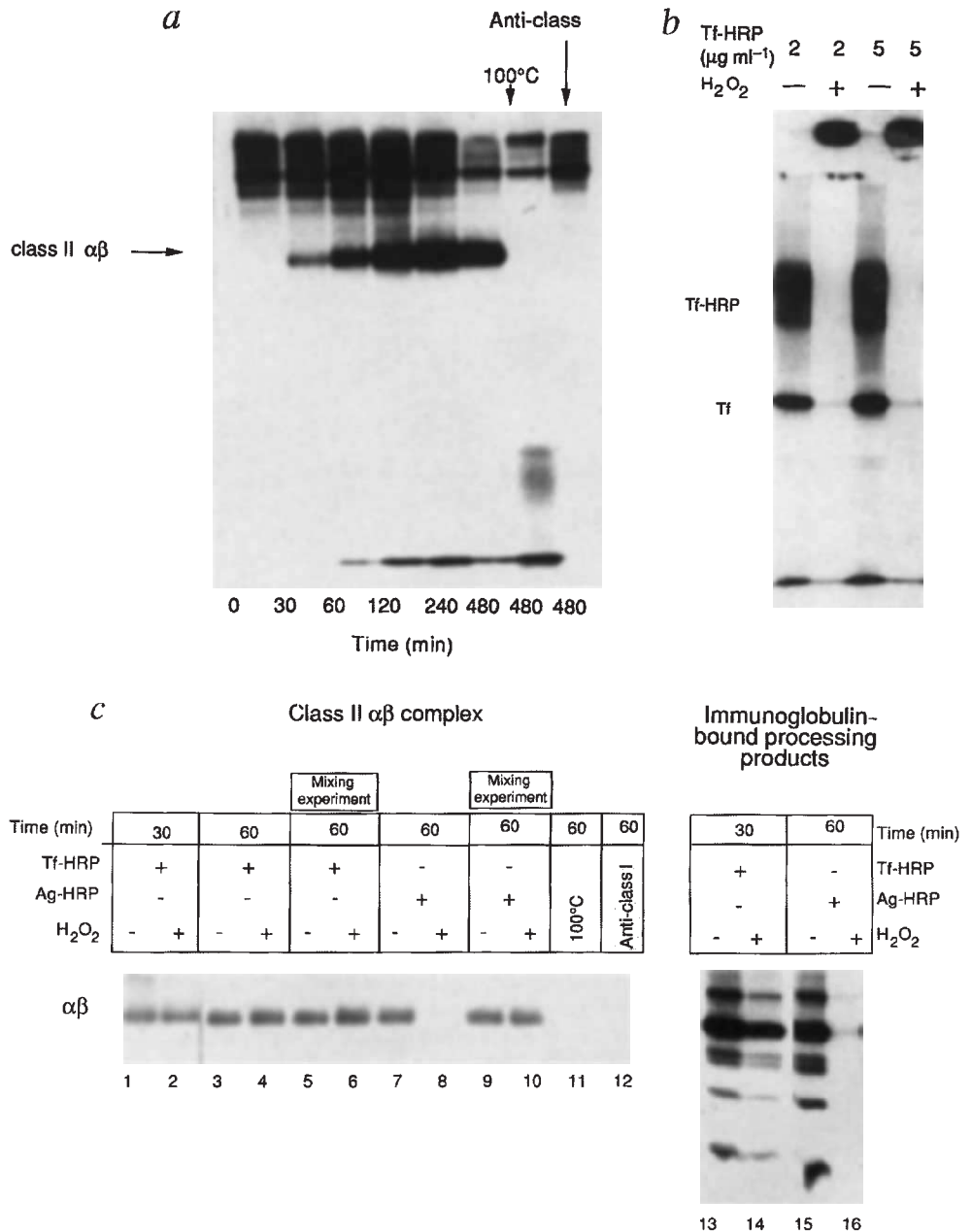
Taken together, these experiments indicate that newly assembled peptide/class II MHC complexes reside in a compartment accessible to antigen through membrane immunoglobulin-driven uptake, but not to endocytosed transferrin receptors. Transferrin receptor-positive early and late endosomes^{19,20} support some antigen processing but are unlikely to be major stations of class II MHC assembly.

To analyse further the compartment containing loaded class II/peptide complexes we fractionated post-nuclear supernatants from antigen-pulsed B cells on 27% Percoll gradients¹⁴. Markers of plasma membrane, early/late endosomes and late endosomes/pre-lysosomes (membrane IgG, internal transferrin receptor and CI-mannose-6-phosphate receptor, respectively) migrated in the low-density fractions (peak density, 1.04 g ml⁻¹), whereas the lysosomal marker β -hexosaminidase peaked at 1.088 g ml⁻¹ (Fig. 2a). During uptake and processing, the ¹²⁵I-label (intact antigen and immunoglobulin-bound fragments) slowly shifted from the low-density plasma membrane/endosome fractions to populate intermediate-density membranes between 30 and 60 min, then denser fractions after longer chase times (Fig. 2b and ref. 14). Class II MHC molecules were immunoprecipitated from pooled pairs of gradient fractions and analysed without boiling to preserve SDS-stable peptide/class II MHC complexes¹⁶. Most of the earliest detectable class II/peptide complexes were found in intermediate density fractions 17–20 (ρ = 1.06 g ml⁻¹) distinct from the plasma membrane/endosome fractions (7–12) and the peak of lysosomal β -hexosaminidase (fractions 23/24) (Fig. 2c, d). As peptide-labelled $\alpha\beta$ complexes accumulated (5.6-fold increase between 30 and 60 min; see Fig. 1a), they appeared in the plasma membrane fractions as expected, but also in progressively denser fractions: ~1.07 g ml⁻¹ (60 min), 1.088 g ml⁻¹ (360 min). Because surface expression is achieved well before comigration with the β -hexos-

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FIG. 1 *In situ* compartmental analysis of antigen proteolysis and class II peptide loading. EBV-B cells were pulsed at 37 °C with ^{125}I -antigen, pre-bound at 0 °C (a), ^{125}I -Tf-HRP (b) or ^{125}I -antigen for times indicated, plus either Tf-HRP (last 30 min) or antigen-HRP (last 60 min) (c). After acid washing to remove cell-surface Tf-HRP, cell aliquots were incubated in DAB solution in the presence or absence of H_2O_2 (b, c). In c, separate populations of cells pulsed with either ^{125}I -labelled antigen or HRP conjugate were mixed before the addition of DAB (lanes 5, 6 and 9, 10). The recovery of ligand was assessed by SDS-PAGE and autoradiography, either directly (b) or after immunoprecipitation and elution in sample buffer with (c, lanes 11 and 13–16) or without boiling (class II complexes in a and c).

METHODS. Ligands: Transferrin and tetanus toxin were conjugated to HRP essentially as described²⁷. 1:1 products were purified by gel filtration on Sephacryl S-200 (Tf-HRP) or S-300 (antigen-HRP) followed by concanavalin A-Sepharose (Pharmacia) affinity chromatography to remove unconjugated antigen. Antigen and HRP-conjugates were iodinated essentially as described¹⁶. The Tf-HRP conjugate recycled with identical efficiency and kinetics to transferrin itself and was confined to the same low-density compartments (see Fig. 2) on 27% Percoll gradients. Compartmental analysis: Clone 8.5 cells were pre-loaded with ^{125}I -labelled antigen, washed and incubated at 37 °C¹⁶. Where indicated Tf-HRP ($10\text{ }\mu\text{g ml}^{-1}$) or antigen-HRP ($7.5\text{ }\mu\text{g ml}^{-1}$) were included in the 37 °C incubation. Cell-surface-bound Tf-HRP was removed by acid washing essentially as described²⁸, with $100\text{ }\mu\text{g ml}^{-1}$ unlabelled transferrin in the final wash. Each sample was resuspended in PBS at a density of 10^7 cells per ml, divided into two aliquots and incubated for 60 min at 4 °C in the dark with DAB ($100\text{ }\mu\text{g ml}^{-1}$; freshly prepared as a 2 mg ml^{-1} stock, 0.22- μm filtered) with or without H_2O_2 (0.02%). The polymerization reaction was stopped by washing in PBS/BSA/5 mM NaN_3 . Recovery of Tf-HRP (b) or antigen processing products (a and c) was assessed following either direct lysis in SDS sample buffer (b) or immunoprecipitation^{13,16} (a and c). In crosslinking experiments, an equal volume of detergent mix was added to the cell lysate to reduce nonspecific adsorption of molecules onto



the DAB polymer¹⁵. Class II MHC, class I MHC and immunoglobulin-associated antigen fragments were immunoprecipitated as described¹⁶ using DA6.231²¹, W6/32 (Serotec) or 312H (anti- λ ; Binding Site), respectively. The immune complexes were eluted in SDS sample buffer and resolved by SDS-PAGE^{13,16}. Autoradiographs were quantified using a phosphorimager (Molecular Dynamics).

aminidase marker (ref. 17 and data not shown), dense lysosomes are not obligatorily involved in the assembly of class II/peptide complexes (Fig. 2d). Fractions enriched in newly assembled complexes (17–22) contained only low amounts of bulk class II MHC as estimated by western blotting of Percoll gradient fractions (Fig. 2d), suggesting transient passage through this vesicle population.

These biochemical data on class II MHC/peptide assembly implicate a transferrin and mannose-6-phosphate receptor-negative compartment apparently distinct from conventional lysosomes. To correlate our data with the ultrastructural study of Peters *et al.*⁹, which identified a class II MHC-rich compartment (MIIC) with a similar marker profile, ultrathin cryosections,

prepared from cells that had internalized 15-nm antigen-gold complexes, were labelled for class II MHC. DA6.231 (anti-DR β -chain antibody²¹) labelled the plasma membrane, small intracellular tubular and vesicular profiles (Fig. 3a) as well as larger structures morphologically very similar to the MIIC compartment⁹. These electron-dense, often perinuclear, spherical structures (200–500 nm diameter) contained arrays of internal membranes which varied from multivesicular (Fig. 3b) to concentric (Fig. 3c) in appearance and accounted for <5% (222 out of 4,800 particles) of total immunogold-labelled class II MHC. Those with six or more particles were defined as MIIC-like.

After 10 min of uptake at 37 °C, 15-nm antigen-gold particles were mainly observed in small, often peripheral, tubules and

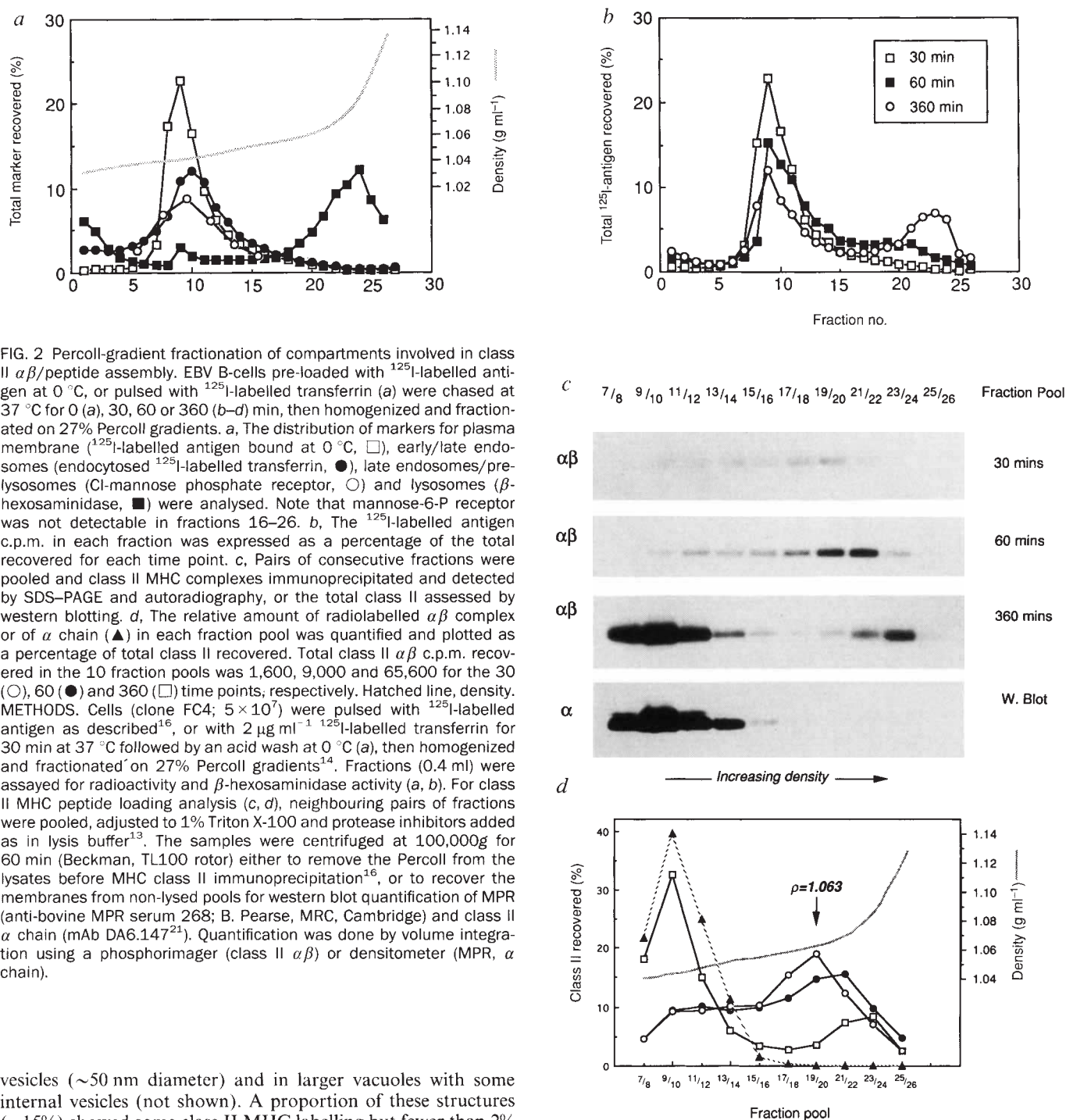


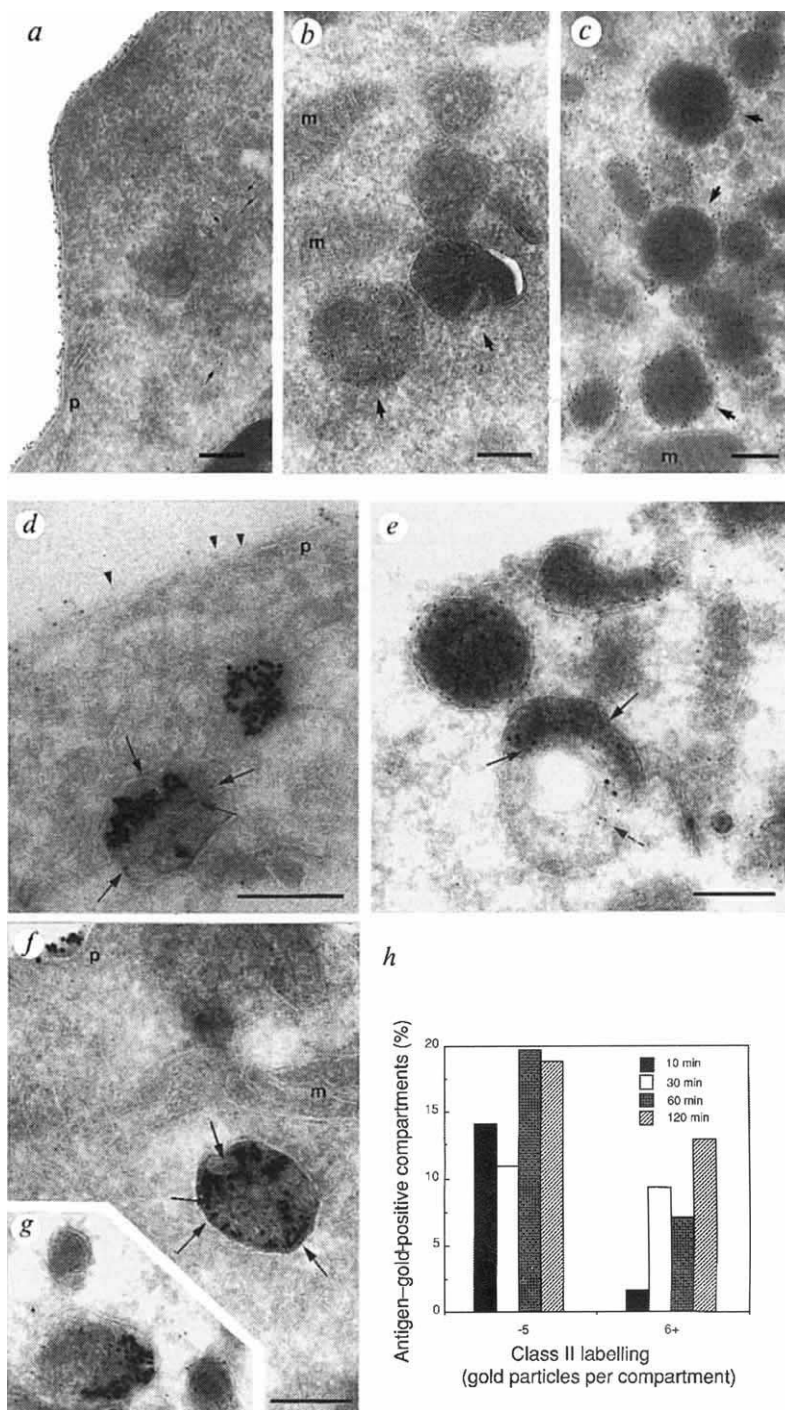
FIG. 2 Percoll-gradient fractionation of compartments involved in class II $\alpha\beta$ /peptide assembly. EBV B-cells pre-loaded with ¹²⁵I-labelled antigen at 0 °C, or pulsed with ¹²⁵I-labelled transferrin (a) were chased at 37 °C for 0 (a), 30, 60 or 360 (b–d) min, then homogenized and fractionated on 27% Percoll gradients. a, The distribution of markers for plasma membrane (¹²⁵I-labelled antigen bound at 0 °C, □), early/late endosomes (endocytosed ¹²⁵I-labelled transferrin, ●), late endosomes/pre-lysosomes (Cl-mannose phosphate receptor, ○) and lysosomes (β -hexosaminidase, ■) were analysed. Note that mannose-6-P receptor was not detectable in fractions 16–26. b, The ¹²⁵I-labelled antigen c.p.m. in each fraction was expressed as a percentage of the total recovered for each time point. c, Pairs of consecutive fractions were pooled and class II MHC complexes immunoprecipitated and detected by SDS-PAGE and autoradiography, or the total class II assessed by western blotting. d, The relative amount of radiolabelled $\alpha\beta$ complex or of α chain (▲) in each fraction pool was quantified and plotted as a percentage of total class II recovered. Total class II $\alpha\beta$ c.p.m. recovered in the 10 fraction pools was 1,600, 9,000 and 65,600 for the 30 (○), 60 (●) and 360 (□) time points, respectively. Hatched line, density. METHODS. Cells (clone FC4; 5×10^7) were pulsed with ¹²⁵I-labelled antigen as described¹⁶, or with $2 \mu\text{g ml}^{-1}$ ¹²⁵I-labelled transferrin for 30 min at 37 °C followed by an acid wash at 0 °C (a), then homogenized and fractionated on 27% Percoll gradients¹⁴. Fractions (0.4 ml) were assayed for radioactivity and β -hexosaminidase activity (a, b). For class II MHC peptide loading analysis (c, d), neighbouring pairs of fractions were pooled, adjusted to 1% Triton X-100 and protease inhibitors added as in lysis buffer¹³. The samples were centrifuged at 100,000g for 60 min (Beckman, TL100 rotor) either to remove the Percoll from the lysates before MHC class II immunoprecipitation¹⁶, or to recover the membranes from non-lysed pools for western blot quantification of MPR (anti-bovine MPR serum 268; B. Pearce, MRC, Cambridge) and class II α chain (mAb DA6.147²¹). Quantification was done by volume integration using a phosphorimager (class II $\alpha\beta$) or densitometer (MPR, α chain).

vesicles (~50 nm diameter) and in larger vacuoles with some internal vesicles (not shown). A proportion of these structures (~15%) showed some class II MHC labelling but fewer than 2% of these were MIIC-like as defined above. Between 10 and 30 min, when processing and class II MHC loading are underway, there was a noticeable fivefold increase in the proportion of antigen-gold-labelled compartments with elevated class II labelling (Fig. 3d, e, h). The MIIC-like structures with irregularly arranged or multi-vesicular internal membranes (Fig. 3d–g) became labelled well before those with pronounced concentric internal membranes. The latter were infrequently labelled with antigen-gold even after 120 min (not shown). These results demonstrate that a subset of MIIC-like structures are accessed by antigen internalized through membrane immunoglobulin and, importantly, within the time frame of peptide loading detected biochemically. Antigen-gold trafficking to the MIIC compartment is likely to be very similar to that of unconjugated antigen because both showed identical kinetics of presentation to T-cell clones (data not shown).

Taken together, our studies demonstrate assembled peptide/class II MHC complexes at a site accessible to internalized membrane immunoglobulin, but distinct from both dense lysosomes and transferrin receptor-positive early/late endosomes^{19,20}. Antigen breakdown, however, may be initiated within the latter domain, consistent with other studies demonstrating that endosomes are proteolytically active^{22–25}. The time-dependent increase in the density of membranes carrying assembled peptide/class II complexes (Fig. 2) might either suggest a progressive maturation of the peptide-loading compartment, perhaps allowing different peptides to be captured and expressed with different kinetics²⁶, or the existence of a subpopulation of

FIG. 3 Trafficking of membrane immunoglobulin-bound antigen-gold and colocalization with class II MHC molecules. *a-c*, Cryosections immunolabelled for class II MHC show labelling of the cell surface, small vesicular and tubular profiles (small arrows; *a*) and larger (>200 nm), heavily labelled structures containing multi-vesicular (*b*) or concentric arrays of internal membranes (*c*). *d-g*, Electron-dense, class II MHC-positive structures, with multi-vesicular or irregular internal membranes become labelled with antigen-gold (15 nm) internalized for 30 (*d*, *e*) or 60 (*f*, *g*) min. Class II MHC (8 nm), arrows; cell-surface WGA-gold (4 nm), arrowheads; m, mitochondria; n, nucleus; p, plasma membrane. Scale bars, 200 nm. *h*, Antigen-gold-positive structures (>100 from each time point), identified in random cell profiles, were categorized into those containing 1–5, or 6+ class II-associated gold particles. WGA-gold-positive structures were taken to be cell-surface invaginations and disregarded.

METHODS. Colloidal gold was prepared by tannic acid-citrate reduction and coupled to ligands²⁹. Cells were incubated with tetanus toxoid-gold (final absorbance at 525 nm, 5.0) at 0 °C for 90 min (labelling was reduced >90% with excess unconjugated toxoid), and chased at 37 °C. The cells were chilled and resuspended in 4 nm WGA-gold (final A_{525} , 1.0) at 4 °C for 60 min. Cell pellets (4×10^6 cells) were fixed for 30 min in 0.5% glutaraldehyde/0.2 M HEPES, pH 7.2, embedded in 2.3 M sucrose, frozen and cryosections prepared³⁰. Grids were incubated with protein A ($10 \mu\text{g ml}^{-1}$ in PBS/0.2% fish skin gelatin) for 10 min, then sequentially in 0.5% glutaraldehyde/PBS and 50 mM NH_4Cl /PBS for 5 min to pre-block endogenous IgG. Class II molecules were detected with mAb DA6.231 followed by rabbit anti-mouse immunoglobulin ($2 \mu\text{g ml}^{-1}$) and protein A-gold and the sections contrasted essentially as described³⁰, before being examined in a Model 1200 EX JEOL microscope. Labelling by DA6.231 was specific in that only cryosections from DR-1-transfected and not untransfected fibroblasts were labelled (kindly provided by L. Lightstone and R. Lechler).



assembled complexes that fail to reach the surface and are instead degraded in lysosomes.

Assembly of class II MHC/peptide complexes outside the domain of conventional (transferrin) receptor recycling indicates that an as-yet uncharacterized trafficking step is necessary for cell-surface expression. □

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Production of soluble MHC class II proteins with covalently bound single peptides

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THE $\alpha\beta$ T-cell receptors (TCRs) react with complex ligands composed of peptides bound to major histocompatibility complex (MHC) proteins. In the absence of foreign antigens the peptides bound to MHC molecules come from the proteins of the host itself^{1–5}. Interactions between TCRs and these self-peptide-MHC ligands work positively to drive T-cell development in the thymus^{6–9} and negatively to delete or inactivate T cells with potential self-reactivity^{10–12}. On the cell surface, MHC proteins are associated with many different self peptides, making it impossible to know which self peptide was involved in positive or negative interactions with a particular T cell. These studies as well as *in vitro* studies on TCR-peptide-MHC interactions would be aided by a means of producing MHC molecules containing a single peptide. We have tackled this problem for MHC class II proteins by genetically attaching the peptide by a flexible peptide linker to the amino terminus of the class II β -chain. Here we report that a secreted, soluble form of this covalent peptide-MHC complex can be expressed in insect cells. The peptide is engaged by the peptide-binding groove of the secreted MHC molecule and this complex is recognized by T cells bearing receptors specific for that combination.

Brown *et al.* recently described the structure of a human DR MHC class II protein with a collection of peptide(s) engaged in its peptide-binding groove¹³. As predicted^{2,3}, the structure showed that the ends of the engaged peptides can project beyond the MHC protein, unlike the case for peptide bound to class I MHC proteins in which the N- and C-terminal ends of the bound peptide are buried in cavities of the MHC cleft^{13–15}. Moreover, the N-terminal end of the MHC class II β -chain (but not α -chain) projects from the side of the molecule into the solvent and is in fact relatively unstructured, because the positions of the first 4 amino acids of the β -chain could not be assigned.

Therefore, we guessed that it might be possible to construct a polypeptide chain in which a peptide covalently attached to the N-terminal end of the β -chain of class II could lie in the peptide-binding groove of the molecule. To test this idea we used a baculovirus system¹⁶ to express in insect cells two murine class II molecules, IE^{dk} and IA^d, with peptides attached by a linker

to the N terminus of their β -chains (as described in Fig. 1). We selected peptides known to bind well to the clefts of these class II molecules, a peptide from moth cytochrome *c* (MCC 91–103)¹⁷ for IE^{dk} (IE^{dk}-MCC) and a peptide from chicken ovalbumin (cOVA 327–339)¹⁸ for IA^d (IA^d-OVA). A thrombin cleavage site was included in the linker sequences. In each case the class II α - and β -chains were truncated to eliminate their transmembrane portions allowing secretion of fully assembled $\alpha\beta$ dimers¹⁶. As controls, similar constructions were made for IE^{dk} or IA^d without covalently attached peptides.

Previous work with full-length naturally produced class II molecules solubilized from cell surfaces with detergent had shown that the stability of the molecule is improved by a bound peptide as determined by increased stability in sodium dodecyl sulphate (SDS)¹⁹. Likewise, baculovirus-produced soluble human DR1 class II molecules are unstable without an associated peptide as determined by stability in SDS and homogeneity and lack of aggregation on high-performance liquid chromatography (HPLC) size filtration¹⁶. Therefore, the four molecules expressed in baculovirus-infected insect cells and purified from culture supernatants by immunoaffinity chromatography were first analysed by HPLC gel filtration (Fig. 2*a, b*). As predicted, IE^{dk} and IA^d without peptide showed high M_r aggregates and apparent size heterogeneity even in the $\alpha\beta$ heterodimer peaks. IA^d appeared particularly unstable with considerable dissociation to free α - and β -chains after elution from the immunoaffinity column.

In contrast IE^{dk}-MCC was greatly stabilized by the covalently attached peptide, migrating sharply as a uniform $\alpha\beta$ heterodimer. Likewise, although IA^d-OVA still formed some high M_r aggregates, it also was considerably stabilized by the OVA peptide with most of the protein migrating as a uniform $\alpha\beta$ heterodimer. This stabilization strongly suggested that the covalently attached peptides occupied the peptide-binding grooves on most of the class II proteins.

We also tested the stability of the IE^{dk}-MCC and IA^d-OVA in 1% SDS (Fig. 3). The IE^{dk}-MCC molecule required heating at 94 °C to dissociate its chains, a result consistent with peptide stabilization of the molecule and similar to that seen with a soluble form of the closely homologous human DR1 molecule¹⁶. In contrast the IA^d-OVA molecule was dissociated by SDS even at room temperature. This result indicated that the IA^d-OVA molecule was less stable than the IE^{dk}-MCC molecule despite its occupancy with a peptide. Because this is the first report of a soluble IA molecule, it is possible that, without the α - and β -chain transmembrane regions, IA molecules are inherently less stable than IE or DR molecules whether or not they are occupied by a peptide.

We examined the peptide-linked class II proteins for their ability to be recognized by T-cell hybridomas. Three MCC peptide IE^{dk}-specific T-cell hybridomas were used and all responded similarly. Data for one of these hybrids, 5KC-73.8, is shown in Fig. 4. The hybridoma responded even better to the covalent IE^{dk}-MCC complex than to purified IE^{dk} subsequently loaded with the MCC peptide (Fig. 4*a*). The hybridoma did not respond to IA^d-OVA, nor to IE^{dk} not loaded with a peptide (Fig. 4*b*). This latter was an interesting control because the IE^{dk} was made in moth cells which could potentially generate the MCC peptide, albeit in a compartment that would probably not be able to provide peptide for loading into class II proteins.

Likewise, an IA^d plus OVA peptide-specific T-cell hybridoma, DO-11.10, responded well to IA^d-OVA (Fig. 4*c, d*). As expected this same hybridoma could not be stimulated by IA^d alone or by IE^{dk}-MCC (Fig. 4*d*). However, the hybridoma also failed to respond to IA^d into which an attempt had been made to load the OVA peptide (Fig. 4*c*). This failure was probably due to the fact that nearly all the IA^d protein dissociated during isolation (Fig. 2*b*).

Two other T-cell hybridomas specific for the same combination of IA^d plus OVA peptide were tested in similar assays. One

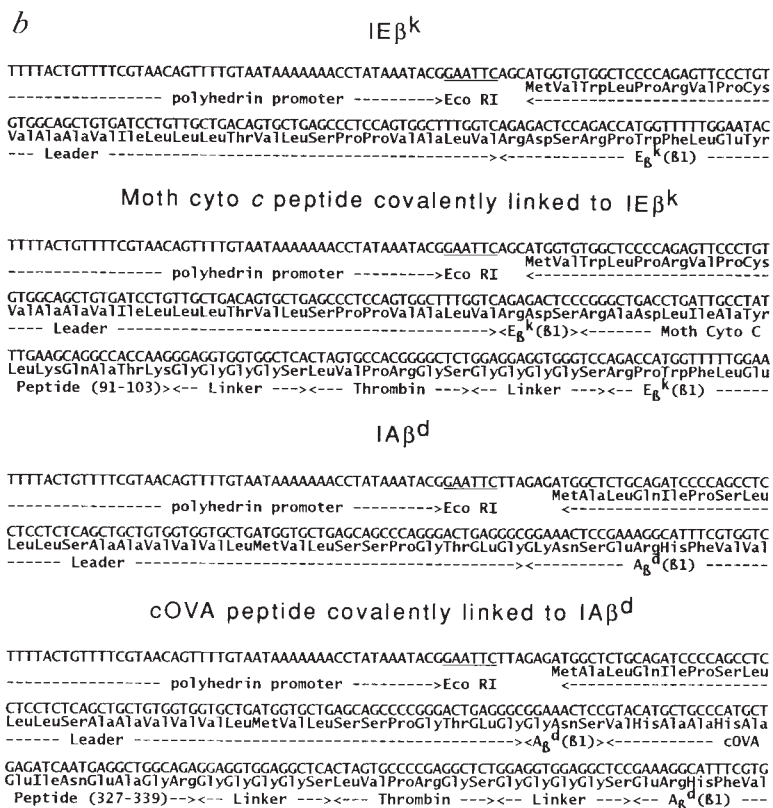


FIG. 1 Expression of murine class II molecule in baculovirus with peptides covalently linked to the β -chain. The dual promoter baculovirus transfer vector, pACUW51 (Pharmingen), was altered using oligonucleotides and the polymerase chain reaction (PCR) to replace both the *EcoRI*-*BglII* sites after the P10 promoter and the *BamHI* site after the polyhedrin promoter with new multiple cloning sites (MCS). The DNA sequences through the new MCSs were:

P10- CACTGATCCTCGAGGGGTGACCGGTCCGGAGGGGTACCAATCCAG

Polyhedrin- AAATACGGAATTCGGGTGACGGAGATCTGGGCATGCGGGGATCCGG.

Two murine MHC class II molecules were prepared. The PCR was used with cloned cDNA templates^{21,22} to produce DNA fragments encoding the IE α^d gene and the IE β^k genes truncated at the last codon before those encoding the transmembrane portion of the proteins and containing restriction enzyme sites for cloning into the MCSs of the new baculovirus transfer vector. Similar versions of the IA α^d and IA β^d genes were produced. The oligonucleotides used were:

5'-TCCTCGAGAAATGGCCACAATTGGAG-3'
 IE α^d XhoI MetAlaThrIleGlyAlaLeu//LeuLeuProGluThrLysGluAsn*** KpnI
 3'-CTTTGATTTCTCTTAATTCATGGTTT-5'

5'-GCGGGAAATTCAGCATGGTGGCTCC-3'
 IE β^k EcoRI MetValTrpLeuProArg//GlnSerThrSerAlaGlnAsnLys*** SphI
 3'-AGACGTGTCTTGTTCATTGTCATCGTACGCC-5'

5'-TCCTCGAGAGGATGCCGTGCAGCAGAG-3'
 IA α^d XhoI MetProCysSerArgAlaLeu//ProMetSerGluLeuThrGluThr*** KpnI
 3'-CTCAGCTGTCTTTGAATTCATGGTT-5'

5'-TACGGAATTTAGAGATGGCTCTGCAGA-3'
 IA β^d EcoRI MetAlaLeuGlnIleProSer//GlnSerGluSerAlaArgSerLys*** SphI
 3'-TCAGACGGGCTCGTTCATTCGTACGCC-5'.

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The IE α^d and IE β^k genes were cloned into the P10 and polyhedrin MCSs, respectively, of a single vector. The IA α^d and IA β^d genes were cloned similarly. In each case the genes were recombined into the Baculogold baculovirus (Pharmingen) in SF9 cells by cotransfection. The initial recombinant virus stock was cloned by infection of SF9 cells at limiting dilution in 96-well plates. A large viral stock of a clone was produced for subsequent large-scale protein production. Versions of these molecules were expressed with a foreign peptide covalently linked to the N terminus of the mature β -chain. For IE β^k the peptide was amino acids 91-103 of moth cytochrome c¹⁷ and for IA β^d it was amino acids 327-339 of chicken ovalbumin¹⁸. a, Strategy schematically superimposed on a representation of the α -carbon backbone of the DR1 molecule¹³. In each case an approximately 100-bp fragment encoding the foreign peptide and a flexible linker with an embedded thrombin cleavage site was introduced in-frame by the PCR just after the third codon of the β 1 domain. This assured a recognizable leader peptide cleavage site and flexible link between the C-terminus of the foreign peptide bound in the cleft of the MHC molecule and the N terminus of β 1 domain, now starting at amino acid 4. b, Nucleotide and protein sequence of the four β -chains from the promoters through the leaders into the β 1 domains.

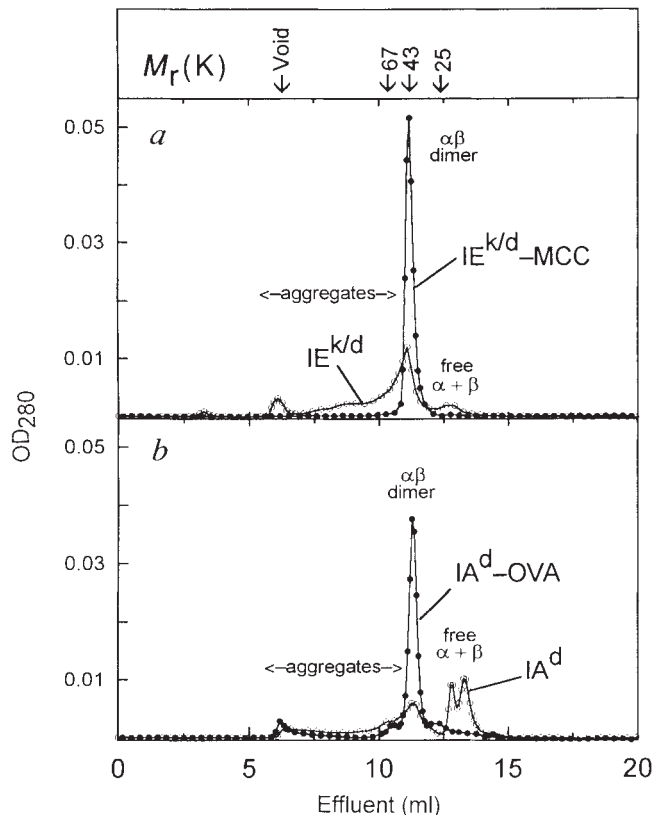


FIG. 2 Stabilization of soluble MHC class II molecules by covalently attached peptides. High 5 insect cells (Invitrogen) at $5\text{--}10 \times 10^5 \text{ ml}^{-1}$ in TMN-FH media were infected at a multiplicity of infection of 5–10 with baculovirus carrying IE^{dk} , IA^{d} , $\text{IE}^{\text{dk}}\text{-MCC}$ or $\text{IA}^{\text{d}}\text{-OVA}$. Five to six days later culture supernatants were collected by centrifugation. The secreted class II molecules were then immunoaffinity purified using immobilized anti- β -chain monoclonal antibodies (14-4-4 for IE^{dk} and M5/114 for IA^{d}). The protein was eluted with a basic buffer at pH 10.5 and immediately neutralized. SDS-PAGE analysis showed in all cases equimolar α - and β -chain in the eluted protein indicating initial secretion of $\alpha\beta$ heterodimers. The overall yield was 0.5 to 1.5 mg l^{-1} of culture media. For gel filtration analysis $\sim 50 \mu\text{g}$ of each protein in $20 \mu\text{l}$ was loaded on a Shodex Protein KW-804 HPLC gel filtration column of dimensions $8 \text{ mm} \times 300 \text{ mm}$ (total volume $\sim 15 \text{ ml}$). The column was eluted at 0.5 ml min^{-1} in PBS and the OD_{280} of the eluate followed. The four elution profiles shown were normalized to the same total OD_{280} . a, IE^{dk} ($\circ$); $\text{IE}^{\text{dk}}\text{-MCC}$ ($\bullet$). b, IA^{d} ($\circ$); $\text{IA}^{\text{d}}\text{-OVA}$ ($\bullet$). The elution positions of M_r standards are shown at the top: BSA, 67K; ovalbumin, 43K; chymotrypsinogen, 25K.

FIG. 4 Stimulation of T-cell hybridomas by purified, immobilized class II/peptide complexes. Purified IE^{dk} (∇), IA^{d} ($\blacktriangle$), $\text{IE}^{\text{dk}}\text{-MCC}$ ($\bullet$), and $\text{IA}^{\text{d}}\text{-OVA}$ ($\blacklozenge$) were prepared as in Fig. 2. We attempted to load synthetic MCC 88–103 into IE^{dk} ($\text{IE}^{\text{dk}} + \text{MCC}$, $\blacksquare$) and synthetic OVA 327–339 into IA^{d} ($\text{IA}^{\text{d}} + \text{OVA}$, $\times$) by incubation overnight at 37°C of $10 \mu\text{g}$ of the class II molecules with $10 \mu\text{g}$ of the peptide in $50 \mu\text{l}$ of citrate buffer at pH 5.0, followed by neutralization and removal of unbound peptide using a Centricon 10 filter unit. In some cases the covalent linkage of the peptides in class II was digested by a 2-h incubation of $10 \mu\text{g}$ of the complex with 2×10^{-3} units of thrombin at pH 6.6, conditions which digested at least 80% of the complex as assessed by SDS-PAGE; thrombin-treated $\text{IE}^{\text{dk}}\text{-MCC}$ ($\star$) and thrombin-treated $\text{IA}^{\text{d}}\text{-OVA}$ ($+$). Different amounts of the various class II preparations were immobilized by overnight nonspecific adsorption to the bottom of wells of 96-well Immulon II plates. Either of two T-cell hybridomas (10^5) were added in $250 \mu\text{l}$ of tissue culture medium. a, 5KC-73.8 ($\text{IE}^{\text{dk}}\text{-MCC}$ -specific)²³ or b, DO-11.10 ($\text{IA}^{\text{d}} + \text{OVA}$ -specific)²⁴. The plates were cultured overnight and the amount of interleukin-2 (IL-2) produced by the hybridomas assessed as previously described²⁵. Results are representative of 2–5 experiments for each of the combinations. In control experiments the MCC and OVA peptides stimulated the appropriate T-cell hybridomas to produce IL-2 when presented by an IE^{dk} - or IA^{d} -bearing antigen-presenting cell, respectively (data not shown).

responded in the same way as that illustrated above. Another hybridoma failed to respond to $\text{IA}^{\text{d}}\text{-OVA}$ regardless of whether or not the linker had been cleaved with thrombin (data not shown). It is possible that recognition of the peptide by the receptor on this T cell hybridoma is context sensitive, that is, it is inhibited in some way by the presence of the few amino acids

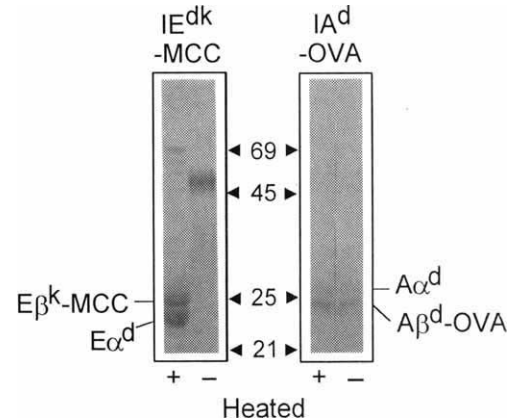
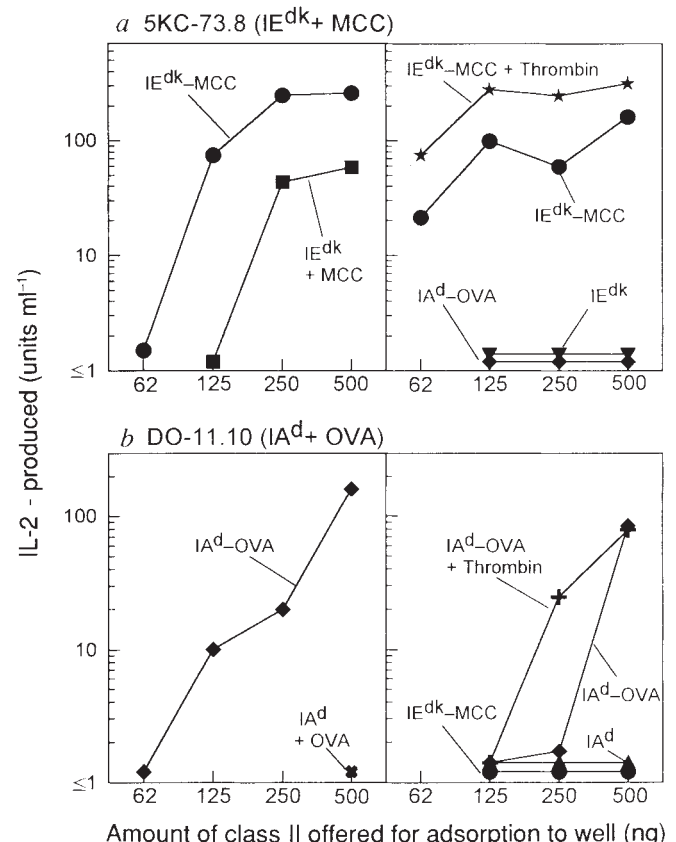


FIG. 3 $\text{IE}^{\text{dk}}\text{-MCC}$ is more stable than $\text{IA}^{\text{d}}\text{-OVA}$. Aliquots of $\text{IE}^{\text{dk}}\text{-MCC}$ ($4 \mu\text{g}$) and $\text{IA}^{\text{d}}\text{-OVA}$ ($2 \mu\text{g}$) were incubated for 5 min in Laemmli sample buffer containing 1% SDS either at room temperature or at 94°C . Samples were then analysed by SDS-PAGE at room temperature. Similar results were seen with IE^{dk} and IA^{d} that had been incubated with the MCC and OVA peptide, respectively, under conditions favouring peptide loading as described in Fig. 4 (data not shown). M_r standards: 69K, BSA; 45K, ovalbumin; 25K, chymotrypsinogen; 21K, soybean trypsin inhibitor. Bands corresponding to the individual chains were identified by comparing $\text{IE}^{\text{dk}}\text{-MCC}$ and $\text{IA}^{\text{d}}\text{-OVA}$ with and without thrombin treatment to IE^{dk} and IA^{d} without a covalently attached peptide (data not shown).



of the $\beta 1$ domain of IA^d on the N terminus of the peptide or the linker amino acids on the C terminus.

In both cases treatment of the peptide-MHC covalent complex with thrombin to cleave the linker between them only modestly improved T-cell hybridoma recognition (Fig. 4b, d). It is therefore likely that usually the linker extends from the C-terminal end of the peptides around the side rather than over the top of the class II α -chain α -helix in order to reach the N-terminal end of the class II β -chain (Fig. 1).

These experiments shows that it is possible to produce a covalent complex of peptide and class II protein which can be recognized by most T cells specific for the combination. Constructions of this type should be useful in experiments on the structure of

T-cell receptor-ligand interactions and in fact, in the case of the IA molecule, may be the only way to generate soluble class II/peptide complexes in reasonable quantities. In preliminary experiments we have found that proteins like this, with their associated transmembrane regions restored, are well expressed on the surface of mouse B cells and fibroblasts where they are recognized by the appropriate T cell and inhibit presentation of other peptides by at least 100-fold (data not shown). The expression by cells of class II proteins bound entirely, or mostly, to a single peptide will be invaluable in studies of positive selection of T cells. They may also allow very efficient induction of tolerance *in vivo* to particular class II/peptide complexes, a phenomenon that may be of therapeutic significance²⁰. □

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Genetically based N-acetyltransferase metabolic polymorphism and low-level environmental exposure to carcinogens

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THE metabolic activation or inactivation of carcinogens varies considerably in human populations, and is partly genetically determined^{1,2}. Inter-individual variability in the susceptibility to carcinogens may be particularly important at low degrees of environmental exposure. Examples of probable human carcinogens that present widespread low-dose exposures are environmental tobacco smoke and diesel exhaust^{3,4}. We have determined levels of DNA adducts in bladder cells and of 4-aminobiphenyl-haemo-

TABLE 1 Levels of ABP-haemoglobin adducts by nicotine-cotinine in 24-h urine and acetylator phenotype in 97 volunteers

	Whole group		Acetylator phenotype		Per cent increase in slow
	Median	Mean	Rapid* (median)	Slow*	
Nicotine + cotinine†					
0 (n=44)	22.5	28.5	13	27	107
<1.5 (n=25)	65	79.5	52	99	90
1.5–2.4 (n=9)	128	123.7	92	132	43
2.5+ (n=19)	125	135.3	128	119	–7

Number of subjects with ABP adducts above/below median

	Acetylator phenotype		OR	95% CI
	Rapid	Slow		
0 (median 22.5)	1/10	21/12	17.5	2.0–153
>0 (median 104)	9/14	18/12	2.3	0.8–7.0

Slow acetylators are those with a 5-acetylaminobiphenyl-3-methyluracil:1-methylxanthine ratio <0.6. The odds ratios (OR) represent the probability of having a high concentration of adducts (greater than the median value) for slow versus rapid acetylators. 95% CI, 95% confidence interval. For the analysis of ABP-haemoglobin adducts, isolated haemoglobin was purified by dialysis and then hydrolysed with base to release the parent amine. After extraction into hexane, the amine was derivatized to form pentafluoropropionamide. Quantification was by comparison of peak areas produced by the analyte and an internal standard (4'-fluoro-4-aminobiphenyl) upon capillary gas chromatography, using negative-ion chemical-ionization mass spectrometry for detection. For determination of the acetylator status, urine samples were collected 5 h after consumption of one standardized cup of coffee. Caffeine and its metabolites were extracted as described⁶. The extracts were analysed by HPLC using a program that separated caffeine and all its known metabolites. Haemoglobin adducts were analysed at MIT, Cambridge; the metabolic phenotype for the NAT polymorphism was determined at NCTR, Jefferson; urinary cotinine and nicotine were measured at IARC, Lyon. * P value for trend <0.0001. † units, μmol per mmol creatinine; n represents number of subjects.

TABLE 2 Correspondence between *NAT2* genotype and acetylator phenotype

Genotype (number of mutations)	<i>n</i>	Acetylator phenotype		OR	(95% CI)	Correlation coefficients: AFMU/1 - X versus nicotine-cotinine
		Rapid	Slow			
0	8	6	2	1.0	—	0.03 (<i>P</i> = 0.9)
1	13	5	8	4.8	(0.7–33)	0.37 (<i>P</i> = 0.2)
2+	20	1	19	57.0	(7.0–460)	0.10 (<i>P</i> = 0.7)
ABP adducts						
	<i>n</i>			Mean	Median	s.e.
0	8			43.2	22	18.5
1	13			68.7	56	17.7
2+	20			69.7	65	12.1

OR, odds ratio; 95% CI, 95% confidence interval; correlation coefficients: AFMU/1 - X ratio versus nicotine-cotinine (*P* values), by genotype; and mean 4-aminobiphenyl-haemoglobin adducts, by genotype (s.e., standard error). The genotype for the *NAT2* gene was determined using PCR and RFLP on DNA extracted from buffy coat. Four mutations have been analysed (M1, M2, M3, M4)⁸. The genotype was determined at the NCI, Bethesda. *n* Represents number of subjects.

globin adducts in 97 volunteers, together with the *N*-acetylation non-inducible phenotype, the corresponding genotype, and the levels of nicotine-cotinine in the urine. We find that among the slow acetylators, 4-aminobiphenyl adducts were higher than in rapid acetylators at low or null nicotine-cotinine levels, whereas the difference between slow and rapid acetylators was less evident at increasing nicotine-cotinine levels. The *N*-acetyltransferase genotype is highly predictive of the acetylation phenotype. Our results indicate that the clearance of low-dose carcinogens is decreased in the genetically based slow-acetylator phenotype. Such genetic modulation of low-dose environmental risks is relevant to 'risk assessment' procedures.

A random sample of smokers (50) and one of non-smokers (50) were enrolled from a population of healthy male blood donors (ages 45–64). After obtaining informed consent, we collected detailed questionnaire data, blood (20 ml) and 24-h urine. After consumption of coffee (1 standardized cup), urine was again collected (5 h). Exfoliated bladder cells were successfully obtained from the urine of 73 subjects, and DNA adducts were examined. The metabolic phenotype for the *N*-acetyltransferase (*NAT*) polymorphism was determined by measurement of caffeine metabolites in 5-h urine. A ratio of 5-acetylaminofluoranthene-3-methyluracil to 1-methylxanthine of 0.6 was used to separate slow from rapid acetylators^{5,6}. The *N*-acetyltransferase *NAT2* genotype was determined using two different polymerase chain reaction (PCR) methods on DNA extracted from buffy coat^{7,8}. Cotinine and nicotine were measured in the urine as markers of recent exposure to tobacco smoke. All phases of the study, including laboratory analyses, were blind. Methods^{5,9} and some results have been described elsewhere^{10,11}.

Table 1 shows the mean and median concentrations of 4-aminobiphenyl (ABP)-haemoglobin adducts by levels of urinary cotinine/nicotine, and by acetylator phenotype. Among non-smokers, 44 had no cotinine or nicotine in the urine, but only 3 had no detectable ABP-haemoglobin adducts in the blood. This observation is consistent with the very low half-life of cotinine and nicotine.

There is a convex dose-response relationship between ABP-haemoglobin adduct levels and increasing cotinine-nicotine levels, a relationship that was previously observed⁹ and parallels a similar relationship for smoking and bladder cancer⁵. Analysis by acetylator status reveals a different distribution. At low cotinine-nicotine levels, slow acetylators include a higher proportion of subjects with high adduct levels. Increasing cotinine-nicotine levels result in a decreasing proportion of slow acetylators with high adduct levels (Table 1). The odds ratio of slow versus rapid acetylators having a concentration of adducts greater than the median is 17.5 among the subjects with no detectable nicotine or cotinine in the urine (95% confidence interval, 2.0–153), and 2.3 (0.8–7.0) among those with nicotine-cotinine greater than zero. Those who had nicotine-cotinine levels equal to zero included 32 subjects not exposed to environmental tobacco

smoke in the 24 h preceding urine collection. Among these, there were 0/9 rapid acetylators with ABP adduct levels higher than the median, whereas the slow acetylators included 9 subjects above the median and 14 below, respectively (odds ratio, infinity). In a multivariate model including four levels of nicotine-cotinine and the acetylator status as independent variables, the difference between slow and rapid acetylators was statistically significant (*T* = 3.2; *P* = 0.0018).

Table 2 shows the results of *NAT2* genotype analysis. DNA was successfully extracted from 82 subjects. In 15, DNA amplification failed because of technical problems. In the remaining 67, a method based on oligo-specific amplification was used⁷. In 41 of these, a second technique based on restriction-fragment length polymorphism (RFLP) was used⁸. Because the latter method was more sensitive and predictive of the phenotype both in this and a previous study⁸, only data for these 41 samples are shown (23 non-smokers and 18 smokers). Four mutations of the gene *NAT2* have been analysed. Only one subject had three mutations, and he had the slow phenotype. The subjects who had at least two mutations had a probability of being slow acetylators 57 times higher than those with no mutation. The correlation coefficients between cotinine-nicotine levels and the 5-acetylaminofluoranthene-3-methyluracil to 1-methylxanthine ratio were not statistically significant within each genotype, suggesting that there was no induction of the enzyme by smoking. The level of ABP-haemoglobin adducts increased with increasing *NAT2* mutations.

TABLE 3 Presence/absence of DNA adducts 2 and 4 in exfoliated bladder cells, by acetylator phenotype in 39 subjects

Presence or absence	Acetylator phenotype	
	Rapid	Slow
Adduct 2		
No	12	17
Yes	3	7
OR (95% CI)	1.6 (0.3–7.8)	
Adduct 4		
No	8	8
Yes	7	16
OR (95% CI)	2.3 (0.6–8.6)	

Adduct 4 is qualitatively similar to *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl. 24-h urine was centrifuged to collect exfoliated urothelial cells on filters and DNA was isolated from cells. ³²P-postlabelling was done under conditions of ATP excess. After autoradiography, individual adducts were visualized, excised and counted. Carcinogen-DNA adduct levels were calculated from the relative adduct labelling:

$$\frac{\text{c.p.m. adducts}}{\text{c.p.m. unadducted nucleotides}} \times 10^9$$

Duplicates (at least) of each sample were analysed separately. DNA adducts were determined at the University of Cincinnati.

Table 3 presents information on DNA adducts in exfoliated bladder cells. Sufficient DNA to detect one carcinogen-DNA adduct per 10^9 normal nucleotides was obtained for 39 subjects (21 non-smokers, 18 smokers). Overall, 12 adducts were found. Two of these (adducts 2 and 4) were moderately associated with smoking habits. Adduct 4 had a correlation coefficient with ABP-haemoglobin adducts of 0.6 ($P=0.01$). This adduct was qualitatively similar to the adduct formed by *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl in the bladders of dogs treated with ABP, and also found in biopsies of human subjects with bladder cancer¹¹⁻¹³. An association between the presence of adduct 2 or 4 in the exfoliated bladder cells and the slow acetylator phenotype is evident, although not statistically significant.

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It is likely that under exceptional exposure conditions (very high levels of exposure) the individual susceptibility is irrelevant. In fact, in a plant where workers were engaged in 2-naphthylamine manufacturing, 15/15 (100%) developed bladder cancer¹⁴. However, low-dose exposure to carcinogens is widespread and its effects are likely to be modulated by genetic susceptibility. For example, environmental tobacco smoke and possibly vehicle exhaust are sources of ABP in non-smokers. Diesel exhaust contains 4-nitrobiphenyl⁴, which is converted to ABP in the body¹⁵. If the population shows genetic heterogeneity in the susceptibility to chemicals, societal and regulatory decisions must be made that focus the risk assessment process on the most susceptible. □

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The stress-activated protein kinase subfamily of c-Jun kinases

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THE mitogen-activated protein (MAP) kinases Erk-1 and Erk-2 are proline-directed kinases that are themselves activated through concomitant phosphorylation of tyrosine and threonine residues¹⁻⁴. The kinase p54 (*M*_r 54,000), which was first isolated from cycloheximide-treated rats, is proline-directed like Erks-1/2, and requires both Tyr and Ser/Thr phosphorylation^{3,5,6} for activity. p54 is, however, distinct from Erks-1/2 in its substrate specificity, being unable to phosphorylate pp90^{rk} but more active in phosphorylating the c-Jun transactivation domain^{5,7,8}. Molecular cloning of p54 reveals a unique subfamily of extracellularly regulated kinases. Although they are 40-45% identical in sequence to Erks-1/2, unlike Erks-1/2 the p54s are only poorly activated in most cells by mitogens or phorbol esters. However, p54s are the principal c-Jun N-terminal kinases activated by cellular stress and tumour necrosis factor (TNF)- α , hence they are designated stress-activated protein kinases, or SAPKs. SAPKs are also activated by sphingomyelinase, which elicits a subset of cellular responses to TNF- α (ref. 9). SAPKs therefore define a new TNF- α and stress-activated signalling pathway, possibly initiated by sphingomyelin-based second messengers, which regulates the activity of c-Jun.

Amino-acid sequences derived from tryptic peptides of purified p54 align with the consensus Ser/Thr kinase catalytic domain¹⁰. Based on these sequences, a 467-base-pair (bp) rat brain complementary DNA probe was generated by polymerase chain reaction (PCR) and used to screen a rat brain cDNA library, yielding four classes of p54 cDNAs (Fig. 1a): α I encoded a protein containing all the tryptic peptides derived from rat liver p54; β and a partial γ -sequence encoded polypeptides closely related to α I (88-90% identity, respectively; Fig. 1a); α II, which probably arises from alternative splicing of the α I transcript, is identical to α I except for a 71-bp region encoding a substitution of 15 amino acids in subdomains IX and X (Fig. 1a and ref. 10). The relative molecular masses of the predicted proteins encoded by the full-length clones are: α I, 48,076; α II, 47,986; and β , 48,095. Northern blots of messenger RNA from several tissues revealed ubiquitous but low expression of all three genes (data not shown).

Alignment of the p54 catalytic domains with those of mammalian and yeast MAP kinases (*KSS1*, *HOG-1*, *FUS3*, *SLT-2*, *spk-1* and *erk-1*; refs 11-14) shows that the degree of homology shared by the p54s with mammalian *erk-1* (43-44%) and with the kinases from lower eukaryotes (41-44%) is almost the same. However, the sequence of *erk-1* is closer to these yeast kinases (49-56% identity) than it is to the p54s. Thus the yeast MAP kinases are unlikely to be functional homologues of the p54s.

p54 isoforms contain the amino-acid sequence TPY at position 183-185, in a domain analogous to the TEY sequence of Erks-1/2 (ref. 15). Erks-1/2 are activated through dual phosphorylation by MAPK or Erk kinases (MEKs)¹⁶⁻¹⁸. But as neither dephosphorylated liver p54 nor bacterially expressed p54 is (re)activated *in vitro* by Erk-1/2-specific MEKs under conditions favouring complete activation of Erks-1/2, the p54s are probably regulated by (a) distinct upstream activator(s).

A polyclonal antiserum raised against the p54 β -isoform immunoprecipitates *in vitro*-translated p54 α and β , but not Erk-1 (Fig. 1b). It also precipitates a cycloheximide-activated GST-c-Jun kinase from NIH3T3 cells (Table 1). We used this p54-specific immune complex assay of GST-c-Jun phosphorylation to investigate the regulation of p54 kinase activity in a variety of cell lines (Table 1a). For comparison, Erks-1/2 activity in the same extracts was assayed, after Mono-Q chromatography, using myelin basic protein (MBP) as a substrate.

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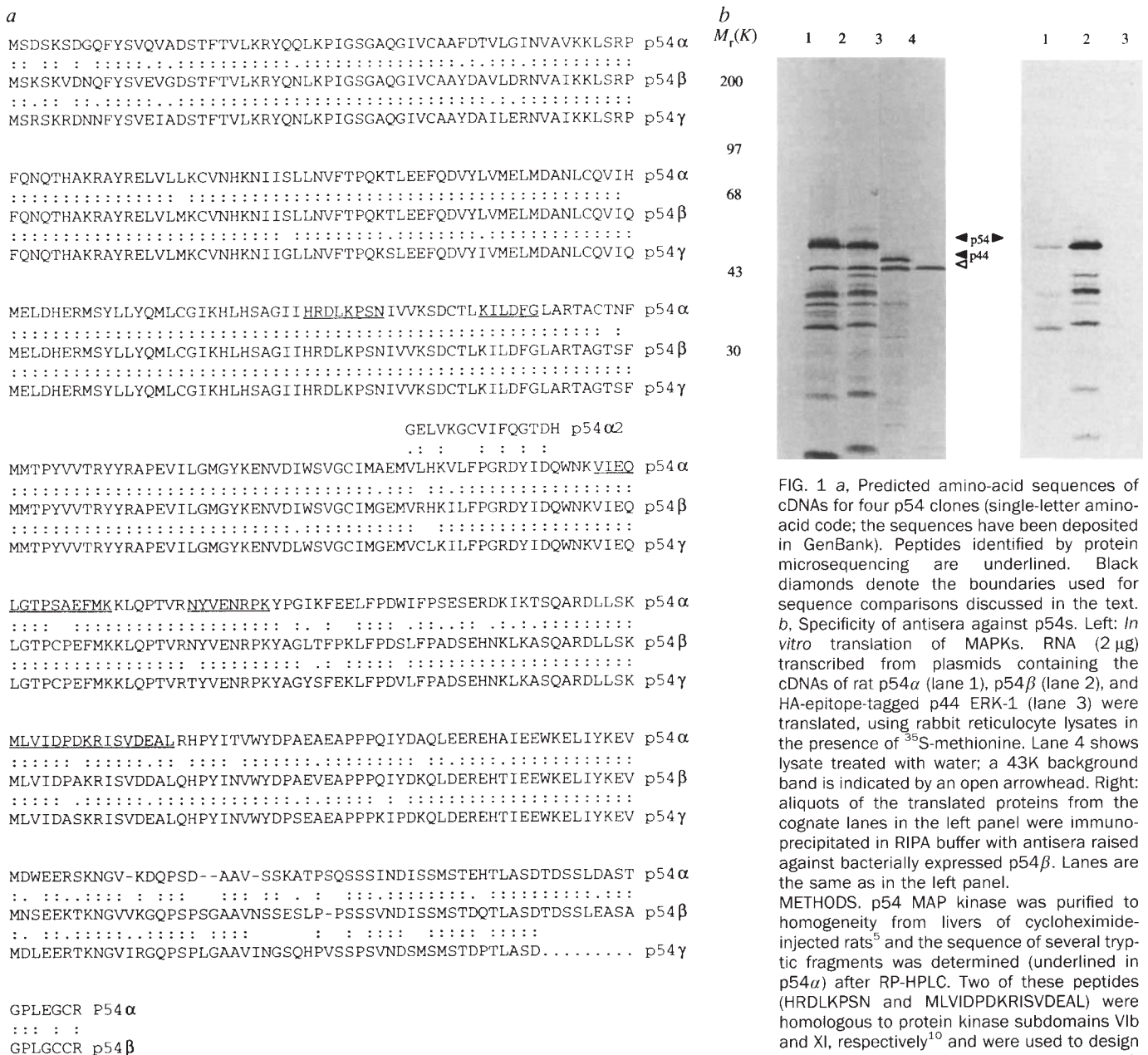


FIG. 1 **a**, Predicted amino-acid sequences of cDNAs for four p54 clones (single-letter amino-acid code; the sequences have been deposited in GenBank). Peptides identified by protein microsequencing are underlined. Black diamonds denote the boundaries used for sequence comparisons discussed in the text. **b**, Specificity of antisera against p54s. Left: *In vitro* translation of MAPKs. RNA (2 μ g) transcribed from plasmids containing the cDNAs of rat p54 α (lane 1), p54 β (lane 2), and HA-epitope-tagged p44 ERK-1 (lane 3) were translated, using rabbit reticulocyte lysates in the presence of 35 S-methionine. Lane 4 shows lysate treated with water; a 43K background band is indicated by an open arrowhead. Right: aliquots of the translated proteins from the cognate lanes in the left panel were immunoprecipitated in RIPA buffer with antisera raised against bacterially expressed p54 β . Lanes are the same as in the left panel.

METHODS. p54 MAP kinase was purified to homogeneity from livers of cycloheximide-injected rats⁵ and the sequence of several tryptic fragments was determined (underlined in p54 α) after RP-HPLC. Two of these peptides (HRDLKPSN and MLVIDPDKRISVDEAL) were homologous to protein kinase subdomains VIb and XI, respectively¹⁰ and were used to design nested degenerate sense and antisense primers, respectively. A 467-bp fragment was amplified by PCR from rat brain cDNA and used as a probe to screen 250,000 plaques from a rat brain cDNA library in λ ZAP (Stratagene). 27 positive plaques were purified and representatives sequenced on both strands using nested deletion series. Positives fell into classes α 1, α 11, β and γ . To generate antisera, the p54- β isoform was subcloned into pGEX-KG and expressed in *E. coli* as a GST fusion protein. The GST-p54 β fusion protein was induced with IPTG (50 μ g ml⁻¹) and purified to homogeneity by glutathione-agarose chromatography, followed by thrombin cleavage to release the kinase moiety; the latter was used as immunogen.

In NIH3T3 and HT-29 cells, Erks-1/2 are strongly activated by mitogens such as EGF, FGF or phorbol ester, and moderately by H₂O₂ or the Ca²⁺ ionophore A23187 (Table 1a). By contrast, the p54s are largely refractory to mitogens and instead are strongly activated by cellular stress. These results indicate that activation of receptor tyrosine kinases or phosphatidylinositol/phosphatidylcholine-directed phospholipases-C does not couple into the activation pathway of the p54 kinases in these cells.

Cycloheximide strongly activates NIH3T3 cell p54s (10-fold), but also gives substantial (5-fold) activation of Erks-1/2 (Table 1a). By contrast, heat shock induces a large increase in p54 activity in NIH3T3 and HT-29 cells but only slight activation of Erks-1/2 (Table 1a).

Polypeptide misfolding is an outcome of both heat shock and translational inhibition; tunicamycin also promotes protein misfolding, but selectively in the lumen of the ER, by inhibiting *N*-linked glycosylation. We found that tunicamycin preferentially activated p54s over Erks-1/2 in HT-29 cells (Table 1a). The data

in Table 1a indicate that cellular stress that promotes polypeptide misfolding activates p54s more effectively than do mitogens.

In mammals, integration of the whole body response to noxious and stressful stimuli is mediated by inflammatory cytokines such as TNF- α and interleukin(IL)-1 β (reviewed in refs 19, 20) TNF- α is a potent activator of the transactivation function and

TABLE 1 Activation of p54, Erks-1/2 and c-Jun-associated kinases

(a)			
Cells	Treatment	p54 Activity (mU)	Erks-1/2 activity (mU)
NIH3T3	Control	5.8 ± 0.6	71.4
	PMA	5.8 ± 0.9	409.7
	FGF	8.5 ± 0.6	795.5
	A23187	8.9 ± 0.7	172.0
	H ₂ O ₂	5.9 ± 0.1	272.2
	Heat shock	38.8 ± 1.7	169.7
	Cycloheximide	57.1 ± 4.1	419.9
HT-29	Control	20.7 ± 1.7	95.0
	EGF	35.7 ± 1.4	576.0
	Heat shock	172.0 ± 28.5	281.0
HT-29	Control	69.4 ± 7.5	195.5
	Tunicamycin	803.9 ± 77.1	480.9
CCD-18Co	Control	7.0 ± 1.0	12.2
	PMA	8.8 ± 0.7	601.0
	EGF	13.0 ± 2.1	394.0
	Heat shock	48.0 ± 1.3	83.7
	TNF- α	60.0 ± 5.0	117.6
Primary porcine hepatocytes	Control	179 ± 13	1,294
	PMA	479 ± 58	3,529
	EGF	665 ± 58	4,882
	Heat shock	697 ± 49	2,427
	TNF- α , 10 min	1,008 ± 31	3,064
HepG2	TNF- α , 20 min	924 ± 38	ND
	Control	29.8 ± 4.3	ND
	TNF- α	434.8 ± 36.4	ND
	Sphingomyelinase	159.0 ± 17.3	ND
(b)			
Treatment	p54 MAPK (mU)	c-Jun-associated kinase (mU)	
Control	31.0	14.5	
Anisomycin	370.0	385.0	
Cycloheximide	166.0	243.0	
Heat-shock	168.0	180.0	
Emetine	75.0	85.0	
Puromycin	19.3	21.5	
Actinomycin D	22.0	24.0	

Confluent cultures of NIH3T3 cells were treated with H₂O₂ (5 mM for 15 min), phorbol-12-myristate-13-acetate (PMA; 500 nM for 20 min), fibroblast growth factor (FGF; 10 ng ml⁻¹, 20 min), A23187 (100 nM, 20 min), heat shock (42 °C, 30 min) or cycloheximide (200 μ M, 60 min). HT-29 cells were treated with epidermal growth factor (EGF; 50 ng ml⁻¹, 20 min), tunicamycin (50 μ g ml⁻¹, 5 h), or heat shock (42 °C, 30 min). CCD-18Co cells (100% confluent) and primary porcine hepatocytes were each treated with PMA (200 nM, 15 min), EGF (50 ng ml⁻¹, 15 min), heat shock (42 °C, 40 min) or TNF- α (50 ng ml⁻¹, 10 or 20 min, as indicated). HepG2 cells (80% confluent) were treated with TNF- α (50 ng ml⁻¹, 15 min) or *S. aureus* sphingomyelinase²⁹ (100 mU ml⁻¹, where 1 U *S. aureus* sphingomyelinase hydrolyses 1 μ mol trinitrophenyl (TNP) sphingomyelin min⁻¹ at pH 7.4, 37 °C). Cell lysis immunoprecipitations were done as described³⁰. Precipitates were assayed for glutathione-S-transferase (GST)-Jun kinase activity as follows. To 40 μ l of a 1:1 suspension of protein G-Sepharose beads containing immunocomplexed p54 were added 20 μ l 0.2 mg ml⁻¹ GST-c-Jun-1-135 (refs 24, 28) or 0.01 mg ml⁻¹ holo-c-Jun^{7,24,28}. [γ -³²P]ATP (100 μ M) and MgCl₂ (10 mM) were added, the reaction was continued for 20 min at 30 °C and stopped with SDS sample buffer before SDS-PAGE. The 40K GST-Jun band was excised and counted by liquid scintillation spectrometry. p54 kinase was assayed in triplicate; means $\pm$ s.d. are shown. Parallel extracts (1 ml) were also chromatographed on MonoQ and fractions assayed for Erks-1/2 MBP kinase activity as described³⁰. A peak of MBP kinase activity always eluted at 200–350 mM NaCl; total Erks-1/2 activity was taken as MBP kinase activity in the combined fractions. (1 U of p54 activity will transfer 1 pmol min⁻¹ of phosphate from ATP to c-Jun; 1 U Erks-1/2 will transfer 1 pmol min⁻¹ phosphate from ATP to MBP). For b, U937 cells were treated with various inhibitors of protein and RNA synthesis: anisomycin (10 μ g ml⁻¹, 30 min), cycloheximide, heat shock (as in a), emetine (10 μ g ml⁻¹, 30 min), puromycin (10 μ g ml⁻¹, 30 min), actinomycin D (10 μ g ml⁻¹, 30 min). Parallel aliquots of extracts were subjected to GST-c-Jun chromatography/assay²⁴ or immunoprecipitation and assay for p54. Mean results for two experiments are shown. ND, not determined.

autoinduction of c-Jun²¹. CCD-18Co colon fibroblasts are acutely responsive to TNF- α ²⁰, which markedly stimulates p54s (Table 1a) whereas EGF and PMA do not. Although TNF- α activates Erks-1/2 in these and other cells, EGF and PMA are far more potent Erk-1/2 agonists than TNF- α (Table 1a). The liver is a major target for TNF- α and EGF/TGF- α ^{19,20}. In primary cultures of porcine hepatocytes, TNF- α preferentially activates p54s rather than Erks-1/2, however, in contrast to CCD18-Co cells, EGF and PMA each comparably activate p54 and Erks-1/2, unlike in CCD-18Co cells (Table 1a).

TNF- α stimulates sphingomyelin hydrolysis and the accumulation of ceramide through activation of one or more sphingomyelinases⁹. Ceramide is a likely second messenger for TNF- α as addition of ceramide derivatives or bacterial sphingomyelinases to intact cells reproduces many of the same cellular responses as TNF- α (ref. 9). TNF- α stimulates p54s in HepG2 cells, as does *Staphylococcus aureus* sphingomyelinase (Table 1a), suggesting that TNF- α -stimulated sphingomyelin hydrolysis may be an early step in the p54-activation pathway.

The p54 kinase phosphorylates the serine residues on the c-Jun polypeptide *in vitro* at positions 63 and 73, sites found on trypsin-digested ³²P-labelled peptides Y and X (Fig. 2b, and refs 7, 22, 23), retarding the c-Jun polypeptide upon SDS-PAGE. If p54s act as c-Jun kinases, then p54 stimulants should also increase c-Jun phosphorylation *in situ* at the X/Y sites. Figure 2a and b shows that c-Jun X/Y phosphorylation in U937 and HepG2 cells is also induced by cycloheximide and heat shock. Moreover, p54s immunoprecipitated from cycloheximide-treated HT-29 cells selectively phosphorylate c-Jun on the X/Y peptides *in vitro* (Fig. 2c).

Strategies that increase c-Jun X/Y phosphorylation also activate c-Jun kinases that bind tightly to immunobilized GST-c-Jun^{24,25}. We therefore investigated whether agonists which activate p54s also activate c-Jun-binding kinases (Table 1b). The parallel activation elicited by heat shock and by RNA and polypeptide synthesis inhibitors (and tunicamycin; results not shown) is striking and suggests that p54s are among the major c-Jun kinases recruited *in situ* by these treatments. However, because Erks-1/2 are also stimulated to some extent by these agonists (Table 1a), the relative contribution of p54 to overall c-Jun kinase activity thus requires more direct measurement.

Accordingly, the contribution of the p54s to total c-Jun N-terminal kinase activity was estimated by immunodepletion of p54 from extracts of TNF- α -stimulated cells before adsorption of the extracts to GST Jun. About 60–70% of total c-Jun N-terminal kinase was removed by anti-p54 antisera over time, with a concomitant increase in c-Jun kinase in the immunoprecipitate (Fig. 2d, left). This depletion of c-Jun kinase is specific insofar as incubation of extracts in the absence of serum or with pre-immune serum gives no decrease in total Jun kinase adsorbing to GST-c-Jun compared with that occurring with anti-p54 serum (Fig. 2d, right). Depletion of c-Jun kinase by anti-p54 antiserum was comparable for extracts from anisomycin-treated U937 cells. Thus, TNF- α - and anisomycin-activated c-Jun X/Y kinases consist primarily of p54s.

We have identified a new subfamily of protein kinases, related to Erks-1/2 but activated preferentially by heat shock, protein-synthesis inhibitors and TNF- α . We have therefore named these enzymes stress-activated protein kinases (SAPKs) on the basis of their presumed function in the cellular response to intra- and extracellular stress. Our results are summarized in Fig. 3. Erks-1/2 are predominantly activated by mitogenic agonists such as EGF, FGF and PMA, which signal through Ras²⁶. In most cells tested, the growth factors that stimulate Ras activate the SAPKs weakly, if at all. Thus, in the CCD-18Co cell, in which EGF fails to activate SAPK despite a large activation of Erks-1/2, Ras is probably not coupled to activation of SAPKs. In those cells, it is likely that TNF- α activation of the SAPKs occurs through another signalling pathway, perhaps involving sphingomyelin hydrolysis.

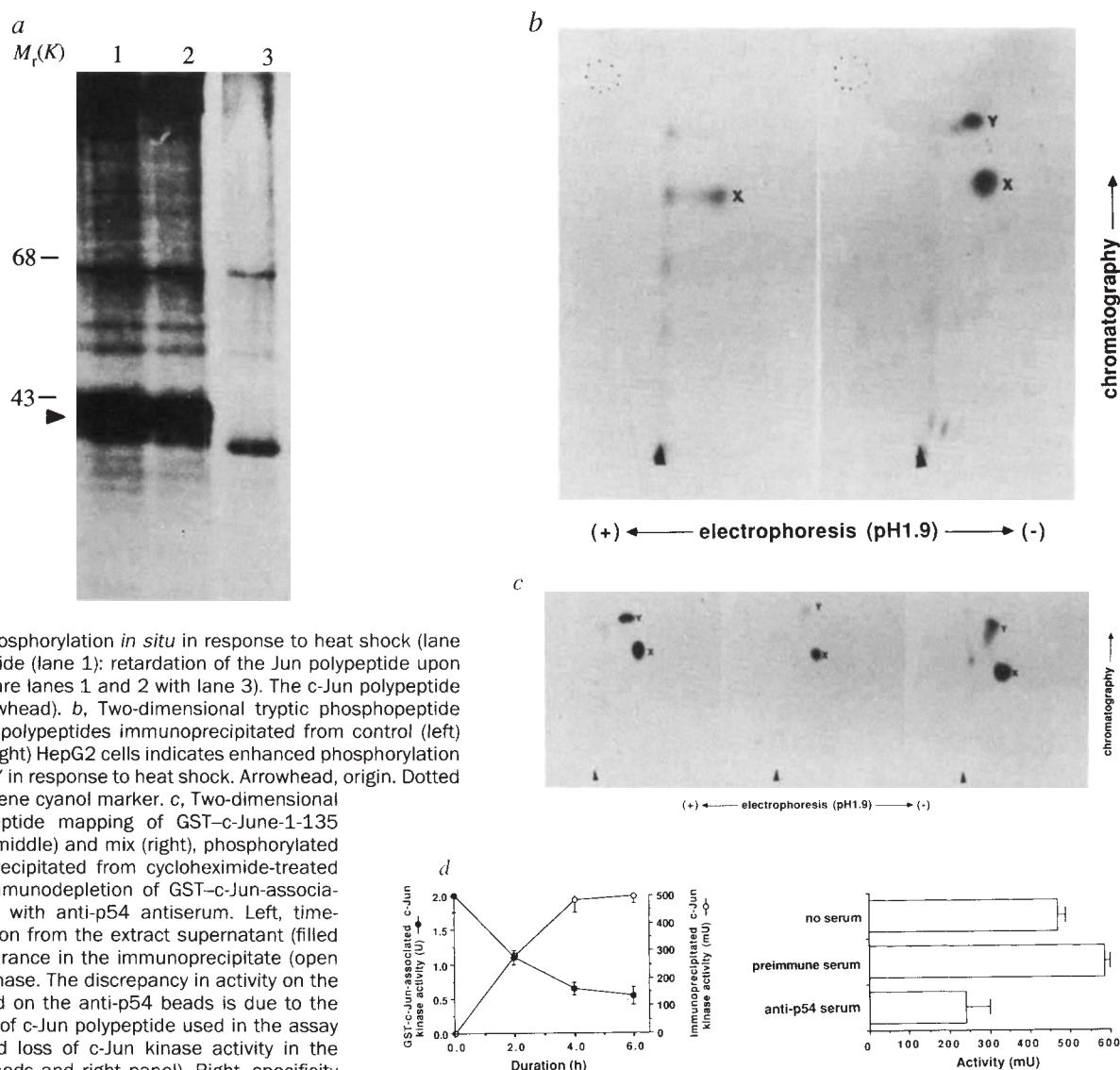


FIG. 2 *a*, c-Jun phosphorylation *in situ* in response to heat shock (lane 2) and cycloheximide (lane 1); retardation of the Jun polypeptide upon SDS-PAGE (compare lanes 1 and 2 with lane 3). The c-Jun polypeptide is indicated (arrowhead). *b*, Two-dimensional tryptic phosphopeptide mapping of c-Jun polypeptides immunoprecipitated from control (left) or heat shocked (right) HepG2 cells indicates enhanced phosphorylation of peptides X and Y in response to heat shock. Arrowhead, origin. Dotted circle indicates xylene cyanol marker. *c*, Two-dimensional tryptic phosphopeptide mapping of GST-c-Jun-1-135 (left), holo-c-Jun (middle) and mix (right), phosphorylated by p54 immunoprecipitated from cycloheximide-treated HT-29 cells. *d*, Immunodepletion of GST-c-Jun-associated c-Jun kinases with anti-p54 antiserum. Left, time-dependent depletion from the extract supernatant (filled circles) and appearance in the immunoprecipitate (open circles) of c-Jun kinase. The discrepancy in activity on the c-Jun columns and on the anti-p54 beads is due to the different amounts of c-Jun polypeptide used in the assay and not to a fixed loss of c-Jun kinase activity in the extracts (see Methods and right panel). Right, specificity of p54 immunodepletion: requirement for anti-p54 serum. Means $\pm$ s.d. for triplicate assays are shown.

METHODS. *a*, U937 cells were labelled with ^{32}P -orthophosphate (1 mCi ml^{-1}) and treated with cycloheximide, heat stress or vehicle (Table 1, legend). c-Jun was immunoprecipitated using a polyclonal antibody specific for the C-terminal 15 amino acids of c-Jun, and subjected to SDS-PAGE. *b*, HepG2 cells were labelled with ^{32}P as in *a* and heat-shocked; c-Jun was immunoprecipitated and was subjected after SDS-PAGE, to two-dimensional tryptic phosphopeptide mapping. *c*, HT-29 cells were treated with cycloheximide; p54s were immunoprecipitated and assayed for Jun phosphorylation. The Jun polypeptide was then subjected to two-dimensional tryptic phosphopeptide mapping. *d*, HepG2 cells were treated with $\text{TNF-}\alpha$ and extracted as described in Table 1 legend. Anti-p54 (left and right panels), blank or preimmune serum (right panel only) were immunoprecipitated for the times indicated (left) or for 4 h (right) (Table 1 legend). Extract supernatants were then subjected to GST-c-Jun chromatography/assay²⁴.

tated and assayed for Jun phosphorylation. The Jun polypeptide was then subjected to two-dimensional tryptic phosphopeptide mapping. *d*, HepG2 cells were treated with $\text{TNF-}\alpha$ and extracted as described in Table 1 legend. Anti-p54 (left and right panels), blank or preimmune serum (right panel only) were immunoprecipitated for the times indicated (left) or for 4 h (right) (Table 1 legend). Extract supernatants were then subjected to GST-c-Jun chromatography/assay²⁴.

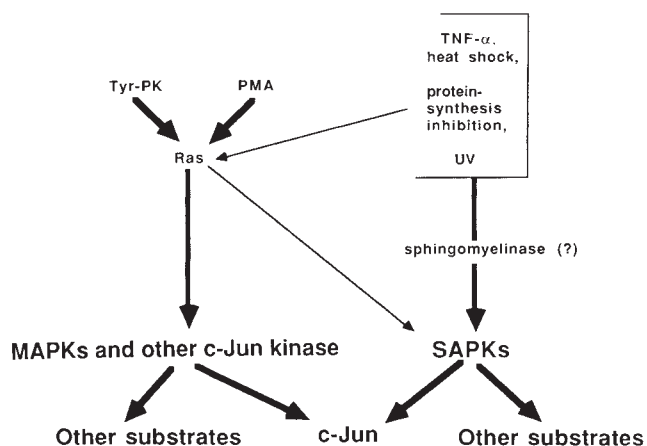


FIG. 3 Hypothetical scheme for mitogenic and stress signalling pathways culminating in phosphorylation of c-Jun and other proteins. Heavy arrows show dominant modes of signal transmission. UV, ultra-violet radiation; Tyr-PK, protein-tyrosine kinase.

The stimuli that activate the SAPKs also stimulate *in situ* c-Jun phosphorylation and/or activation, and, in the case of TNF- α , our results suggest that the SAPKs are the dominant c-Jun kinases. The SAPKs thus join an expanding group of c-Jun N-terminal kinases which focus regulators such as phorbol esters, Ras, ultraviolet radiation^{7,22,27,28}, and now heat shock, translational inhibition and TNF- α into phosphorylation of the c-Jun transactivation domain.

Note added in proof: DNA sequence analysis of additional SAPK clones done after submission of this report identified several cDNAs identical to SAPK- β through S379, after which a 5-nucleotide insertion shifts the reading frame, resulting in the sequence AQVQQ-stop. The kinase reported by Dérjard *et al.*³¹ is identical in sequence to the SAPK- γ cDNA reported here through L379 but then terminates in AQVQQ as does our shorter SAPK- β cDNA. □

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Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment

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THE polyomaviruses are non-enveloped, icosahedrally symmetrical particles with circular double-stranded DNA genomes^{1,2}. The outer shell of the virion contains 360 copies of viral protein VP1 ($M_r \sim 42K$) arranged in pentamers³. We report here the structure at 3.65 Å resolution of murine polyomavirus ('polyoma') complexed with an oligosaccharide receptor fragment. This structure has been determined using the previously described model of simian virus 40 (SV40)⁴. Although very similar in structure to SV40, polyoma has interesting biological differences. Cell-surface *N*-acetyl neuraminic acid (sialic acid) is required for polyoma infectivity, but not for SV40. Polyoma attaches to the surface of susceptible cells by stereospecific recognition of oligosaccharides terminating in (α 2,3)-linked sialic acid^{5,6}. Studies of pathogenicity show that the specificity of viral binding to such oligosaccharides is an important determinant of the virus' ability to establish a disseminated infection and to induce tumours in the natural host. The complex described here shows how polyoma recognizes the receptor fragment and how strains with different receptor specificities can distinguish between alternative ligands. The results also suggest an explanation for the large disparity in pathogenicity exhibited by strains differing in only one amino-acid residue of VP1^{7,8}.

The receptor specificity of polyoma strains has been analysed using attachment to red blood cells (haemagglutination) as an assay. An (α 2,3) linkage of sialic acid (NeuNAc) to galactose (Gal) is essential⁶. Small plaque strains can tolerate attachment of a second sialic acid by (α 2,6) linkage to *N*-acetyl galactosam-

ine (GalNAc) in the following structure: NeuNAc-(α 2,3)-Gal-(β 1,3)-(NeuNAc-(α 2,6)-GalNAc. 'Large plaque' strains cannot bind to such a branched oligosaccharide. The critical difference between small and large plaque strains is the presence of glycine or glutamic acid, respectively, as residue 91 in VP1⁷. Otherwise isogenic strains differ not only in their plaque morphology but also in their ability to cause tumours in mice. The small plaque strains produce few, if any, tumours; the large plaque strains are highly tumorigenic⁸. Our crystals of the small plaque strain P16 diffract to 3.65 Å resolution, and we have been able to diffuse 3'-sialyl lactose (NeuNAc-(α 2,3)-Gal-(β 1,4)-Glc) (Glc = glucose) into these crystals to obtain a complex. We note that, as expected, 6'-sialyl lactose does not bind. 3'-Sialyl lactose is a good model for the terminal parts of likely mammalian cell-surface ligands, such as NeuNAc-(α 2,3)-Gal-(β 1,3)-GalNAc, NeuNAc-(α 2,3)-Gal-(β 1,3)-GlcNAc or NeuNAc-(α 2,3)-Gal-(β 1,4)-GlcNAc⁹. The structure determination is summarized in the caption to Fig. 1, which shows electron density for the trisaccharide. Details of the structure determination will be reported elsewhere. The refinement used data that extend to only moderate resolution. Nevertheless, the results obtained show that the model is in very good agreement both with experimental data (low 'free' *R*-factor) and with standard geometry. The final averaged electron density map is of high quality, and the detailed similarity of the six independent VP1 coordinate sets in one icosahedral asymmetric unit shows that our model is sufficiently accurate for the interpretations that follow.

An overview of a polyoma subunit, with bound trisaccharide, appears in Fig. 2a. The view is essentially the same as the view of the SV40 subunit in ref. 4. The only significant conformational differences between VP1 of SV40 and polyoma are confined to surface loops connecting the β -strands of their 'jelly-roll' framework. Polyoma VP1 contains 22 more amino acids than SV40, of which 18 are found in the BC, DE, EF and HI loops (see ref. 4 and Fig. 2a for notation). The larger BC loop in polyoma can clearly be divided into two segments, labelled BC1 and BC2. The sialic acid lies in a shallow groove between loops BC1 and HI (Fig. 2b); the groove is closed off at one end by the tip of loop BC2 from the clockwise neighbouring subunit in the pentamer. The plane of the sugar ring lies roughly parallel to the walls of the groove. The carboxylate faces toward BC1, and the glycerol moiety faces toward DE. The galactose lies at the

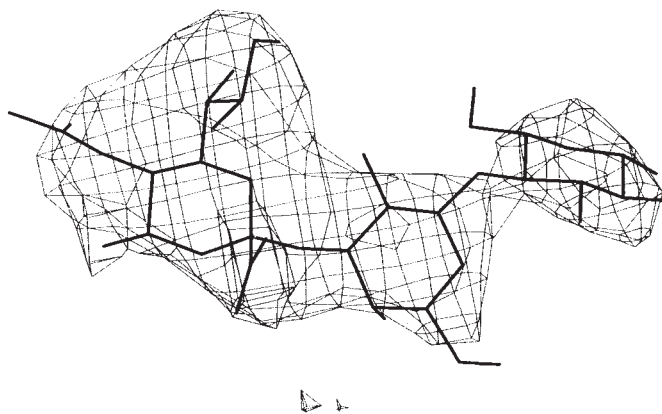


FIG. 1 5-fold averaged electron density map for the 3'-sialyl lactose. The density is detailed enough to position and orient all three sugar rings. The map was calculated using $(2F_{\text{obs}} - F_{\text{calc}})$ Fourier coefficients between 12 and 3.65 Å resolution. Phases were calculated using the refined model and then further improved by 5-fold non-crystallographic 'icosahedral' averaging. The map is contoured at 1.0σ . For reasons of clarity, only contours within 1.5 Å of the model are plotted. The refined model of 3'-sialyl lactose is shown. The conformation of the trisaccharide is similar to the conformations seen in other protein/3'-sialyl lactose complexes^{12,14}. Map and model were obtained as follows: virus (Strain P16) was purified using a slightly modified version of the protocol described in ref. 15. Crystals were grown by hanging drop vapour diffusion. Drops contained 6–8 mg ml⁻¹ virus, 10 mM HEPES pH 7.5, 0.25–0.3 M sodium sulphate and 2.5% (v/v) glycerol; the reservoir contained 10 mM HEPES pH 7.5, 0.5–0.6 M sodium sulphate and 5% (v/v) glycerol. Virus crystals grew within 2 to 4 weeks to a maximum size of $400 \times 600 \times 600 \mu\text{m}^3$. The crystal space group is I23 with $a = 570$ Å; there are 30 VP1 molecules in the asymmetric unit. Crystals were soaked for 20 h in collection buffer (0.65 M sodium sulphate, 5% (v/v) glycerol, 50 mM HEPES pH 7.5) containing 20 mM 3'-sialyl lactose (Accurate Chemical & Scientific Corp., USA). Soaking did not noticeably perturb the crystal packing. Diffraction data were recorded on imaging plates (Fuji Inc., Japan) using an oscillation camera at the F1 synchrotron beamline of CHSS (wavelength 0.91 Å) and scanned using a Fuji

BAS2000 scanner. The crystals were cooled to -10°C during data collection; the oscillation angle was 0.2° with an average exposure time of 30 s per image. Only one image per crystal volume was recorded. Images were integrated with the program DENZO (Z. Otwinowski, Yale University). Further data reduction used the CCP4 program suite¹⁶. The final data set consists of 241,457 unique observations obtained from 87 images. The data are 73.2% complete; the merging R -factor is 11.9% (12 to 3.65 Å). All three sugars of the 3'-sialyl lactose could be localized clearly in a difference Fourier $((F_{\text{soak}} - F_{\text{nat}})\exp i\alpha_{\text{nat}})$ -map calculated at 3.8 Å resolution, where 'nat' denotes previously collected data obtained from 'native' uncomplexed crystals and refined phases from the corresponding averaging procedure using these data; the electron density for the sialic acid was the highest positive peak in the entire map. The previously determined structure of uncomplexed polyomavirus was improved by several rounds of 5-fold non-crystallographic averaging¹⁷ and intermittent rebuilding with FRODO¹⁸. The crystallographic R -factor for the unrefined model was 45% (12–3.8 Å). Rigid body refinement using XPLOR¹⁹, with 5-fold non-crystallographic icosahedral symmetry as a strict constraint, reduced the R -factor to 44.7%. A test set of 2% (4,826 reflections) of the data set was excluded from refinement and used to calculate a 'free' R -factor²⁰. Several rounds of positional and temperature factor refinement with XPLOR (12 to 3.65 Å), followed by manual rebuilding produced a model with good stereochemistry (r.m.s. bond and angle deviations from ideal values: 0.02 Å and 2.7° , respectively). The final R -factors are 23.5% (working set) and 25.3% (free set) for all available data in the resolution range 12–3.65 Å. Polyoma VP1 contains 383 amino-acid residues. The first 16 of these are not visible and have been excluded from our model, as well as the C-terminal residues which do not assume ordered structures in some of the VP1 molecules. The use of the strict non-crystallographic constraint for the 5-fold icosahedral symmetry proved to be very powerful both for driving the refinement to convergence and for assessing the quality of its result. Because there are 30 molecules in the asymmetric unit, but only a strict 5-fold symmetry restraint was applied during refinement, we could assess the quality of the refinement by a comparison of the 6 independently refined VP1 molecules. When omitting the CD-loops and the C-terminal arms, which assume different structures⁴, the α -coordinates of the six VP1 molecules can be superimposed with a r.m.s.-deviation of 0.5 Å. Plots of thermal parameters versus residue numbers are (again with the exception of the CD-loops and the C-terminal arms) strikingly similar for all six molecules. Together with the low free R -factor, this similarity shows that the refinement indeed produced a structurally sensible result.

base of loop BC2, which forms an extended 'plateau' supported by part of loop EF. Loop DE of the anticlockwise neighbouring subunit approaches this plateau from the rear. The glucose ring lies above the plateau, but there are few if any van der Waals contacts to protein atoms. The thermal parameters for the glucose are higher than those for the sialic acid or galactose, consistent with the lack of strong constraint. Its orientation seems to be determined by a hydrogen bond between Glc-O3 and Gal-O5.

The shape of the binding groove is complementary to a preferred conformation of the NeuNAc-(α 2,3)-Gal-linkage¹⁰. This conformation is determined not only by potential steric hindrance for many choices of torsion angles about the glycosidic bond but also by a hydrogen bond from Gal-O2 to NeuNAc-O6. A series of polar interactions between amino-acid side chains and groups of the sialic acid and galactose appears to orient the ligand further. Included in the description that follows are those hydrogen bonds for which the donor-acceptor distances are below 3.5 Å in all six independently refined coordinate sets. One key interaction appears to be a salt bridge between the carboxylate of the sialic acid and the guanidinium group of Arg 77. There are no negatively charged residues in the vicinity to neutralize the arginine, and it is possible that the electrostatic field generated by its uncompensated charge has a role in attracting and orienting the receptor. The nearby side chain of His 298 may enhance the charge field. The 'rear wall' of the groove is distinctly hydropho-

bic, with the side chain of Val 296 in direct contact with the plane of the sugar ring. Additional hydrogen bonds link the phenolic oxygen of Tyr 72 to the sialic acid amide group and His 298 to NeuNAc-O4. There appears to be only one hydrogen bond to the galactose, between Asn 93 and Gal-O6. Gly 78 is probably also important for accommodating the galactose, because even a small side chain at this position would collide with Gal-O4.

Influenza virus also uses sialoglycoconjugates as receptors, and the structures of the influenza haemagglutinin in complex with various α -sialosides have been determined^{11,12}. The primary binding site is a shallow pocket on the outer surface of the haemagglutinin. Only the sialic acid makes clear contacts, and both 3'- and 6'-sialyl lactose can bind. There is no homology with the interactions seen in the polyoma/sialyl lactose complex, but the number of hydrogen bonds is comparable. Both polyoma and influenza have quite low affinities for individual receptors (the dissociation constants are in the range of 1 mM) but when a virus attaches to a cell, more than one receptor is engaged, and a strong cooperative interaction occurs. By contrast, the tight binding of sugars by transporter-associated proteins involve very extensive hydrogen bond networks and very precisely complementary interaction¹³.

It is possible to model related oligosaccharides into the polyoma site. In addition to the two pockets which accommodate the NeuNAc (α 2,3) Gal moiety, we can discern a pocket for the

(α 2,6) linked sialic acid of the branched-chain receptor (see Fig. 3). A preliminary analysis of crystals containing such a branched oligosaccharide shows that the second sialic acid is indeed located close to this pocket (T.S. and S.C.H., unpublished results). In one of the sterically favourable conformations of the branched linkage⁹, the carboxylate of this sialic acid approaches the space

where the side-chain carboxylate of glutamate 91 would be in large plaque strains. We therefore believe that coulombic interference can explain why large plaque strains do not bind branched-chain receptors.

Small plaque viruses, capable of recognizing branched as well as straight-chain sialyloligosaccharides, may bind more avidly

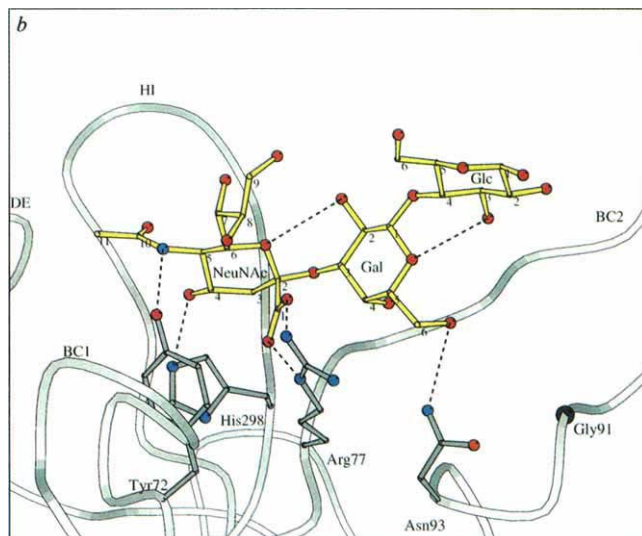
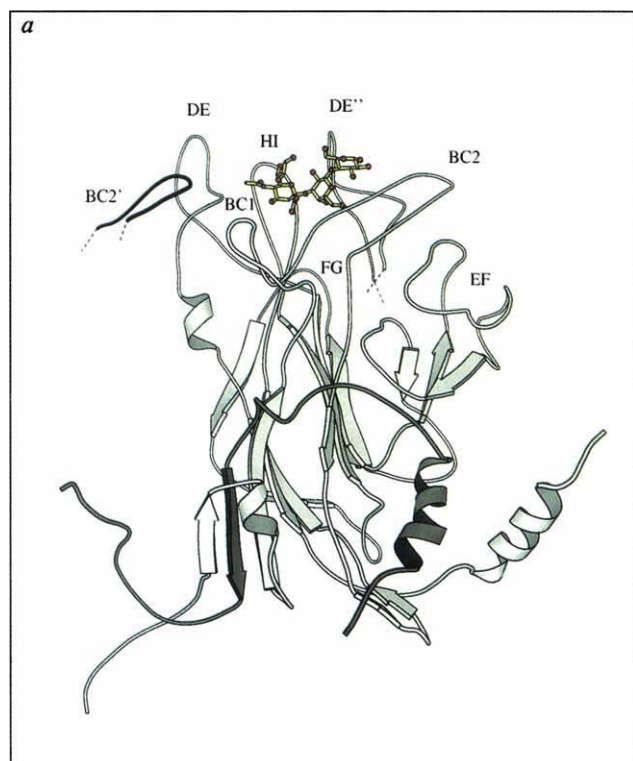


FIG. 2 The receptor binding site of polyomavirus. *a*, Schematic drawing of a refined polyomavirus VP1 monomer together with its receptor analogue 3'-sialyl lactose, shown as a ball-and-stick model. The loops that form the binding site are labelled. Residues 78 to 89 of the BC2-loop of the clockwise neighbour and residues 137 to 159 of the DE-loop of the anticlockwise neighbour are also shown. The C-terminal arm that belongs to another pentamer and invades VP1 is shown in dark shading (see ref. 4). *b*, Enlarged view of the receptor binding pocket. The side chains of Tyr 72, Arg 77, Asn 93 and His 298 (in grey) as well as the trisaccharide (in yellow) are shown as ball-and-stick models. The α -atom of Gly 91 is marked with a black sphere. Hydrogen bonds are given as broken lines. *a* and *b*, Generated with MOLSCRIPT²¹. In the ball-and-stick models, oxygen atoms are red and nitrogen atoms are blue.

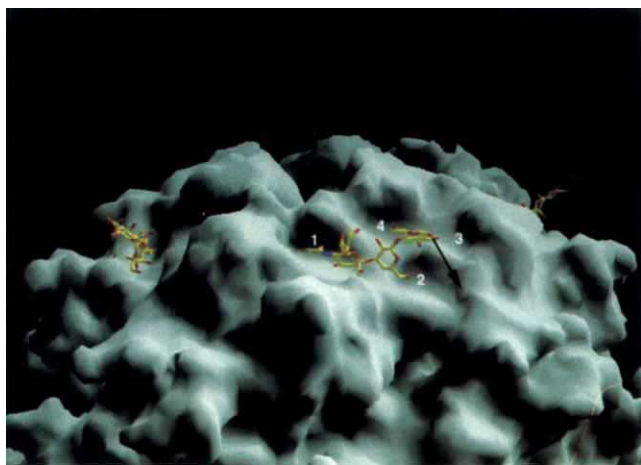


FIG. 3 Surface representation of a complete VP1 pentamer, with the 3'-sialyl lactoses shown as ball-and-stick models. Four pockets (numbers 1–4) are evident. Pockets 1 and 2 accommodate sialic acid and galactose, respectively. Pocket 3 could accommodate the (α 2,6)-linked sialic acid of a branched-chain receptor (see text). Although the function of pocket 4 has not yet been investigated, it is conceivable that it accommodates sugar moieties of still more complex oligosaccharide receptors. The arrow shows the position of the linkage to other sugars in complex oligosaccharides or to protein in short O-linked glycans. The figure has been generated with GRASP (A. Nicholls & B. Honig, Columbia University).



FIG. 4 View onto the virion surface, centred at a pentacoordinated VP1 pentamer (shown in green) on a strict icosahedral 5-fold symmetry axis. The VP1 molecules are represented with α -atom tracings, the corresponding trisaccharides are shown in a solid atom representation (carbon, yellow; oxygen, red; nitrogen, blue). The figure was generated with O²².

to cultured mouse cells by virtue of interaction with both types of oligosaccharides on the cell surface. It is therefore somewhat paradoxical that the small plaque strains, with broader oligosaccharide binding capacity, are effectively inhibited from spreading in the intact host⁸. A possible explanation for the lower pathogenicity of small plaque viruses would be that sialoglycoconjugates rich in branched chains are present in tissues of at least some mouse strains, and that such structures act effectively as 'pseudoreceptors' or inhibitors specifically of these viruses. The relative densities of branched and straight-chain sialoglycoconjugates present on surfaces to which the virus can bind may also play a role. The distribution of VPI on the virus surface in principle allows simultaneous binding of many receptors without significant membrane distortion (Fig. 4), but the receptors would need to cluster within a region of 200–300 Å in diameter. The large plaque viruses may thus escape from inhibition by virtue of 'ignoring' surfaces with a high density of branched sialoglycoconjugates. □

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ERRATUM

A relict refractory inclusion in a ferromagnesian chondrule from the Allende meteorite

Keiji Misawa & Takashi Fujita

Nature **368**, 723–726 (1994)

IN this letter, part of the legend to Fig. 2 was omitted. The penultimate sentence should read:

“The spinel exhibits Fe–Mg zoning: the core and the Ca, Al-rich silicate inclusions are iron-free, whereas in the rim (~20–40 µm width) and along the cracks the FeO concentration rises sharply (MgO ≈ 4 wt %). The silicone inclusions are composed of two phases: a Ca, Al-rich phase (low MgO content) and Ti, Al-pyroxene, fassaite.” □

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Reading between the genes

The most extensive comparison so far of contiguous genomic DNA in humans and mice reveals a surprising degree of conservation in intergenic sequences.

WHATEVER the true number of genes in the human genome — let's say 75,000 give or take 20,000 — the burning question remains as to what function (if any) the often vast lengths of DNA that have no direct purpose for encoding peptides might serve. These mysterious sequences, which may make up as much as 97% of the human genome for example, range from apparently barren stretches of highly repetitive DNA threading the individual genes together to the numerous sequences known as introns that punctuate the coding regions of most genes.

Over the years, many people have come to regard these inter- and intragenic curiosities as simply 'junk' DNA, evolutionary relics that can tolerate endless amounts of change (polymorphism) without any untoward effect on the host organism. Of course, there are a few exceptions. Some genes are clearly regulated in part by crucial DNA motifs that may sit many kilobases from the gene itself, but maybe these are just the exceptions that prove the rule.

Or are they? As the amount of DNA sequenced from humans, mice and other organisms grows, it is becoming possible to compare matching pieces of DNA from different species to discern the amount of sequence conservation between them. In the geneticist's creed, evolutionary conservation is a key indicator of functional importance, even if that precise role is not immediately apparent to the hapless investigator. Until recently, such comparisons have been handicapped by the limited amount of contiguous genomic DNA sequence available. But with the continuing improvements in automated DNA sequencing technology, prospects are looking up.

Writing in the May issue of *Nature Genetics*, Ben Koop and Leroy Hood (*Nature Genet.* 7, 48–53; 1994) describe their scrutiny of almost 100 kilobases of homologous human and mouse genomic DNA sequence from the T-cell receptor locus. T-cell receptors are membrane-bound heterodimers that closely resemble antibodies and are made up of two

different pairs of polypeptide chains, either α/β or γ/δ . In addition to constant (C) regions, a series of variable (V), diversity (D) (in the case of the γ/δ chains) and junction (J) segments contribute to the impressive variability exhibited by different receptors.

Koop and Hood focused on the 100-kilobase C α /C δ regions of mouse and human, which by their calculation contain 50 and 61 J α segments, respectively. Together, these coding regions account for only 6% of the total DNA. Of course, it is not surprising that these genes should show a high degree of conservation — of the order of 75% in fact. But, surprisingly, a survey of the entire region, including 94% non-coding DNA, produces an astonishing similarity value of 71% between mouse and human. This is the highest figure seen so far and contrasts with the results of other reasonably large sequence comparisons performed previously, such as those for globin or crystallin gene clusters.

As yet there are no firm answers to explain this degree of sequence conservation over such a large expanse of DNA. In the authors' view, it is probably the result of natural selection acting within and beyond the confines of coding elements, supporting the notion of chromosomes as 'information organelles' which perform additional functions such as the storage, copying and evolution of information. Of course, there may be more mundane explanations. Further large-scale sequence comparisons will be required to make the distinction.

Human and mouse homologies are being exploited in other ways, notably in the search for genes controlling complex traits, which can obviously be performed more easily in fast-breeding mice with controlled genetic backgrounds. One promising area is the search for genes underlying behavioural traits, including the tendency to abuse substances such as drugs and alcohol. In another report in this month's issue, Wade Berrettini and colleagues describe some potentially important genetic differences between two mouse strains which have a vastly different preference for morphine (*Nature Genet.* 7, 54–58; 1994).

When given the choice between a bottle of sweetened quinine (the sweetener helps to remove a bitter aftertaste) and a suspension of morphine, the C57Bl/6J inbred mouse will happily consume as much suspension as 300 mg per kg per

day, resulting in a characteristic tail muscle contraction called Straub tail and severe withdrawal symptoms. By contrast, the DBA/2J mouse can be tempted to take 10 mg per kg per day, but no more. These dramatic differences are genetically controlled, and thus by performing a quantitative trait loci analysis that has successfully been used to map murine loci for hypertension, cancer and epilepsy, Berrettini and colleagues have been able to identify the positions of three genes responsible for most of the variation in morphine consumption.

Their approach was simply to breed a second-generation intercross between the two strains, and then type the animals with the highest and lowest morphine consumption with some 150 microsatellite markers. In so doing, they detected three loci on mouse chromosomes 10, 6 and 1 (in order of significance) that together appear to account for almost 90% of the total genetic variance. As in humans, the addictive behaviour of the C57Bl/6J mice crosses pharmacological boundaries — that is, they are also partial to substances such as alcohol and cocaine. The search is now on to see whether genes in the homologous human chromosomal regions may explain human patterns of substance abuse.

One gene that will be found before then — although it is taking longer than expected — is the hereditary breast and ovarian cancer gene, *BRCA1*, on the long arm of chromosome 17. About 5% of breast cancer cases among women are inherited, and because many would argue that the disease is reaching epidemic proportions, that makes its familial form one of the most common inherited disorders of all. Men, too, contract breast cancer. Its incidence is 100 times less than in women, but are inherited forms of male breast cancer also attributable to *BRCA1*? The answer at this stage appears to be that they are not. A large consortium study, led by Michael Stratton, has looked at 22 families and found no evidence to support linkage of male breast cancer to the *BRCA1* locus (*Nature Genet.* 7, 103–107; 1994). It would seem, then, that at least one other important gene is accountable for these cases, just as *BRCA1* is evidently not the only gene responsible for hereditary breast cancer in women.

Kevin Davies

Kevin Davies is the Editor of Nature Genetics.

Also in May's *Nature Genetics*: gene defects in male pseudo-hermaphroditism and stationary night blindness; *p53* mutations in anaplastic Wilms' tumour; radiation hybrids and 'genomic sequence sampling' for whole genomes; and *VHL* mutations in renal carcinomas.

DNA snippets

Products from the molecular biology marketplace include nuclear extracts for gene regulation studies, a kit for purifying DNA from tissue and mouse tails, and one for the *in vitro* packaging of DNA from bacteriophage lambda.

EXPANDING its new transcription product line, Trevigen announces the availability of **HeLa cell and *Drosophila* nuclear extracts** for gene regulation studies (*Reader Service No. 100*). The company's *Drosophila* extract production protocols were developed in conjunction with Drs Kamakaka and Kadonaga of the University of California, San Diego. Nuclear extracts are a good source of RNA polymerase II transcription factors for both *in vitro* transcription and DNA binding studies. Trevigen says that important features of its nuclear extracts include their ability to support both basal and activated transcription from a variety of promoters. In addition, both HeLa and *Drosophila* extracts are also good sources of sequence-specific DNA binding proteins. Suggested applications include studying promoter strength, mapping mRNA start sites, synthesis of RNA for primer extension or S1 nuclease analysis, western immunoblotting, DNA mobility shift assays and DNase I footprinting.

Transduction Laboratories has a new line of **monoclonal antibodies to Ras signaling pathway proteins** (*Reader Service No. 101*). The line-up includes antibodies to Ras, GAP, NF1, GAP-associated p190, Rap1, Rap2, Rap-GAP, CDC25 and Sos. The company provides quality control data for western blot, immunoprecipitation and immunofluorescence analyses. Positive control cell lysates for western blotting are available for each antibody. Also of interest is the company's **monoclonal antibodies to several protein kinase C isoforms**. These include protein kinase C- α , - β , - δ , - ϵ and - θ . Again, quality control data for western blot, immunoprecipitation and immunofluorescence analyses are provided, along with the appropriate control cell lysates.

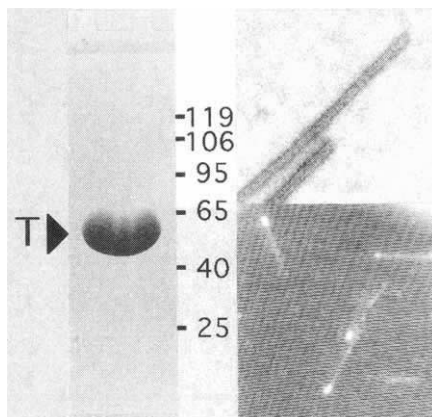


Immunofluorescence microscopy using p190 monoclonal antibody on human fibroblasts.



Immunofluorescence microscopy using anti-PKC delta on human fibroblasts.

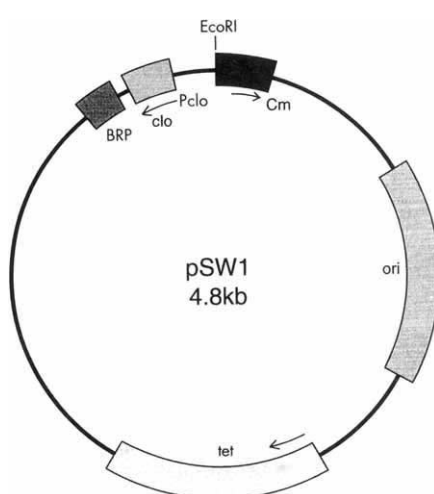
Bovine **brain tubulin** is now available from Cytoskeleton (*Reader Service No. 102*). The protein is said to be greater than 99 per cent pure and with a biological activity of greater than 97 per cent. Tubulin is supplied in PIPES buffer, either plus or minus 10 per cent (v/v) glycerol. Fluorescently labelled tubulin is also available. Labelling is achieved



SDS-PAGE gel of tubulin protein, left; electron micrograph of microtubules, right; and fluorescently labelled, polarity marked microtubules, bottom right.

using an activated ester of the fluorochrome which reacts with random surface lysines (stoichiometry of dye per heterodimer is 1 to 1). Pure tubulin can be used to study the dynamics and biochemistry of microtubules, to examine the activities of known microtubule-associated proteins (MAPs) such as tau, MAP2, kinesin and dynein, and to identify new MAPs. A further area of interest involves the use of tubulin in screening for anti-cancer drugs such as taxol and vinblastine. Also available from Cytoskeleton are several kits for the study of microtubule-protein interactions.

MoBiTec's **BRP vectors** express the bacteriocin release protein (BRP), resulting in a permeabilization of the outer and inner membranes of *Escherichia coli* (*Reader Ser-*



BRP vectors: for controlled protein release into the cell culture medium.

vice No. 103). As a consequence, soluble cytoplasmatic and periplasmatic proteins are released into the culture medium. The system is designed for small- as well as large-scale biotechnological production. The BRP vectors pJL3 and pSW1 are co-transformed with the recombinant vector containing the gene for the protein of interest. The BRP gene is induced with IPTG (pJL3) or mitomycin C (pSW1). BRP activates phospholipase A of the outer membrane resulting in the formation of permeable zones in the cell envelope. The overproduced recombinant protein is released and purified directly from the culture medium. The cells are left intact. Consequently, MoBiTec says that protein purification on a large-scale basis in continuous culture is facilitated: problems with the lethality of recombinant proteins, as well as their preferential inclusion body formation are avoided, and the possibility of degradation by endogenous proteases is reduced. The vector system can be used in combination with the company's pAX5 vector, a system for fusion cloning and protein purification via APTG affinity columns.

Isolation

The Nucleobond AX L-Kit from The Nest Group is designed for the **isolation of undegraded, ultrapure lambda DNA** from a lysate in less than 4 hours, without the need for DNase I, RNase A or phenol (*Reader Service No. 104*). Lambda DNA appears as one sharp band on agarose gels (lane 1), with, according to the company, no smearing from partial degradation and no genomic DNA bands (lane 2). The kit has been used for restriction mapping, subcloning, amplification and sequencing. Each is supplied with ready-to-use, gravity-flow cartridges and buffers. Kits can also be obtained for plasmid DNA purification.



Nuclease-free lambda DNA kit.

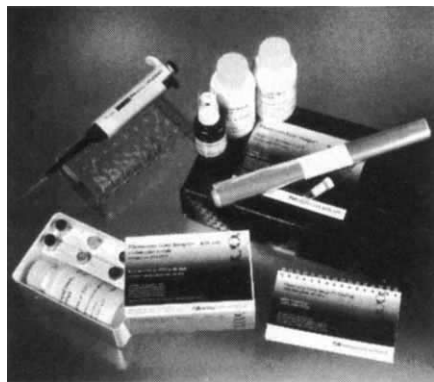
Qiagen's **QIAamp blood and tissue kits** are designed for the purification of DNA from a variety of sources for reliable amplification by PCR (*Reader Service No. 105*). The spin-column procedure requires no cell separation, organic extraction or alcohol precipitation, and is suitable for processing multiple samples. Tissue samples are lysed in 1-3 hours, without homogenization, says Qiagen; blood and cells require 10 min. In

PRODUCT REVIEW

10 min more, the DNA is said to be free of haemoglobin and other contaminants, and ready for direct use in amplification reactions or applications using Southern blotting. The QIAamp blood kit can be used to purify DNA directly from whole blood, plasma, body fluids and cultured cells. This kit should also prove useful in purifying viral RNA from plasma or serum for RT-PCR. The QIAamp tissue kit is suitable for all of the above types of samples, as well as human and animal tissue, paraffin sections and mouse tails. Also of interest is the company's Ni-NTA spin kit, which provides a method for the **screening and purification of 6×His-tagged proteins** from small-scale expression cultures. Each Ni-NTA spin column can purify up to 100 µg of 6×His-tagged protein to up to 95 per cent homogeneity under native or denaturing conditions, says Qiagen. The microspin procedure allows 12 minipreps to be processed in parallel in under 30 min. The company also offers QIAexpress pQE high-level **bacterial expression vectors** for N- or C-terminal placement of the 6×His tag in all reading frames of recombinant proteins.

■ Detection

Fluorescein Gene Images is Amersham's new **nonradioactive nucleic acid labelling and detection system**, intended for applications requiring high sensitivity (*Reader Service No. 106*).



Amersham's Fluorescein Gene Images kit is said to provide sub-picogram nucleic acid detection levels.

Using a fluorescein-11-dUTP method to label DNA, detection is achieved by an alkaline phosphatase/dioxetane (based on Lumi-Phos 530, Lumigen Inc.) system. This is said to allow single copy genomic Southern blots (with as little as 0.1 pg DNA) to be detected. In addition, because the light output is said to last for up to 7 days, multiple exposures can be taken.

DNase contamination can be a concern in molecular biology laboratories that work with DNA. With this in mind, Mo Bio Laboratories has introduced a **DNase detection kit** for determining and quantifying the presence of DNase in your liquid sample or experiment (*Reader Service No. 107*). An

incubation and agarose gel electrophoresis run results in detection of DNase. Extracts can be made from laboratory plastics for detecting DNase on solid surfaces. The company also offers a contract DNase testing service.

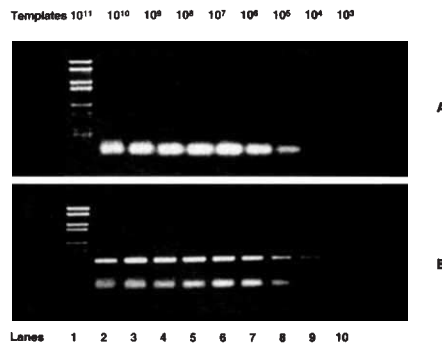
Oncor's two **probes** for the identification of chronic myelogenous leukaemia (CML) and acute lymphocytic leukaemia (ALL) for *in situ* hybridization on metaphase and interphase spreads of paraffin-embedded tissue are now available from Alpha Laboratories (*Reader Service No. 108*). The Philadelphia chromosome [t(9;22)(q34;q11)], which has been observed in CML and some cases of ALL, is characterized by the fusion of the *bcr* gene on chromosome 22 and the *abl* oncogene on chromosome 9. It has been determined that the *bcr/abl* translocation can occur at either of two alternate sites. The major breakpoint region, M-*bcr*, is the site of translocation in CML and approximately 50 per cent of Ph + ALL cases. The minor breakpoint region, m-*bcr*, is proximal and is the site of translocation in the remaining 50 per cent of Ph + ALL cases.

Superior quantum yield, binding affinity and fluorescence enhancement are some of the stated features of the two new **fluorescent dyes for the detection of DNA and RNA**, SYBR Green I and II gel stains, now available from Molecular Probes (*Reader Service No. 109*). Low intrinsic fluorescence of the SYBR Green stains means that gels stained with either dye can be viewed directly, without destaining prior to photography. With SYBR Green I, Molecular Probes says that less than 100 pg of DNA can be detected using a standard ultraviolet transilluminator; even better results are said to be obtained using 254 nm epi-illumination. SYBR Green I also stains single-stranded oligonucleotides in polyacrylamide or agarose gels with about 100 times the sensitivity of ethidium bromide, according to the company. SYBR Green II RNA gel stain is intended for use in the detection of single-stranded nucleic acids. As little as 100 pg of rRNA can be detected using 254 nm epi-illumination. This stain should prove useful in the detection of viroid RNAs and other multicopy cellular RNAs, as well as single-strand conformation polymorphism analysis.

The Optitran **transfer membrane** matrix available from Schleicher & Schuell is made of pure nitrocellulose, reinforced by an inert, ultrathin fleece (*Reader Service No. 110*). S & S says that the immobilizing membrane layer is cast evenly on both sides of the reinforcing fleece, showing the same binding and background characteristics as nitrocellulose — but Optitran is said not to tear. Stated applications include western blots using multiple detection, colony hybridization and plaque lifting.

■ Kits

Boehringer Mannheim's PCR Core Kit ^{PLUS} is designed to **eliminate troublesome DNA template carry-over** (*Reader Service No. 111*). This kit is said to combine the specificity and high yields of the company's PCR Core Kit with the ability of uracil-DNA glycosylase (UNG) to prevent carry-over



Amplification of varying amounts of dU-containing β-actin template a, with and b, without UNG treatment; lane 1, DNA MWM VI.

contamination in polymerase chain reactions. The new kit is guaranteed to yield 10⁵ copies in a standard function test using a DNA fragment as template. Besides giving high yields of a specific amplified DNA, it is designed to prevent contaminants from previous polymerase chain reactions from producing false positives in subsequent DNA amplification experiments. Each lot is tested using an amplification mixture with 10⁵ copies of dUTP-containing DNA as template. Boehringer Mannheim says that no carry-over contamination of dUTP-containing DNA is observed.

The Ready-To-Go **lambda packaging kit** from Pharmacia Biotech is intended for the *in vitro* packaging of DNA from bacteriophage lambda (*Reader Service No. 112*). It



Ready-To-Go lambda packaging kit.

provides seven single-dose lambda packaging extracts, supplied in a convenient, dry format that can be stored at room temperature. To use, add the solution containing the intact lambda DNA resulting from previous cDNA or genomic cloning experiments to begin the packaging reaction. Each reaction



KiloBase DNA marker.

is said to provide a packaging efficiency of greater than or equal to 1×10^8 plaque-forming units per microgram of control lambda DNA (which is also provided in the kit). Also of interest is the KiloBase DNA marker for sizing linear double-stranded DNA fragments. The marker consists of 11 bands from 0.5 to 10 kilobases. The uniformly spaced bands are said to stain with approximately the same intensity. Except that is for the band at 1 kb, which is three times as intense as the others to provide a quick check of the gel's orientation.

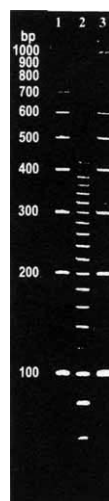
The Interchange system from Promega is designed to provide a quick alternative to both library and standard site-directed mutagenesis procedures for studying the effect of amino acid replacements on protein structure and function. (*Reader Service No. 113*). Using this system, up to 12 amino acids may be replaced at any location within a protein by making only one codon change within the cloned gene. The system is based on the placement of an amber stop codon (TAG) at a position of interest within a cloned sequence, by a site-directed mutagenesis method such as the Altered Sites II System. The mutant plasmid is introduced into one or more of the 12 *Escherichia coli* strains supplied; each carry a different amino acid suppressor tRNA. During expression, the suppressor tRNAs recognize the amber stop codon and insert the selected amino acid at that location. Promega says that this approach has been used to find sites that are important for protein structure and function in the *lac* repressor and T4 lysozyme. (In the case of T4 lysozyme, 163 positions were mutated, and the results of 2,015 amino acid substitutions obtained.) All 12 amber suppressor strains and the control strain supplied with the Interchange system are low-efficiency competent cells. Two control DNAs are also included.

■ Markers

The TransDye tris-buffered dye solution available from Integrated Separation Systems is a highly visible marker for the gel dye front on a western blot (*Reader Service No. 114*). Loading 2 μ l of TransDye into a well when the sample is loaded produces a

vivid pink spot at the dye front that, unlike most tracking dyes, will transfer to, but not through, the transfer membrane, ISS says. After transfer, the visible pink spot, which also fluoresces under ultraviolet illumination, serves as a mark of the bottom of the gel lane, simplifying correct placement in a mini-blotter device or for cutting the membrane into strips.

GenSura Laboratories is offering a new DNA sizing marker, the Superladder-low, which extends to 1 kilobase — the ideal range for polymerase chain reaction products — and is two ladders in one kit (*Reader Service No. 115*). The 100-base pair (bp) ladder (lane 1) consists of ten, and the 20-bp ladder (lane 2) of fifty, sharp bands detected by ethidium bromide staining with very low background, GenSura says. The 100-bp ladder can be used alone, or combined with the 20-bp ladder, providing an easy-to-read ruler-like appearance composed of darker main divisions and lighter subdivisions (lane 3). The 20-bp ladder is intended to bring an increased level of accuracy to routine agarose or polyacrylamide gel sizing of PCR- or restriction-generated DNA fragments. Superladder-low is derived from a cloned and purified 1,000 bp DNA fragment, with no unwanted vector DNA band. It is supplied as separate tubes of 100-bp ladder and 20-bp ladder, modified $\times 6$ gel-loading buffer, and a prelabelled empty tube for storing the combined 100-bp/20-bp ladders. The 20-bp and 100-bp ladders can also be ordered separately.



A 100-bp/20-bp ladder kit for sizing of PCR and restriction-generated DNA products.

■ Hardware

EasyJect from Stratagene is an all-in-one electroporation system that consists of an electroporation chamber, a single high-voltage capacitor, a remote keyboard, and three programmable RAM cards for creating, running and storing customized protocols (*Reader Service No. 116*). Two voltage ranges are offered and nine resistor settings to produce optimal pulse lengths for all cell types.



EasyJect — the all-in-one electroporator.

For example, the high-voltage range is suitable for recalcitrant bacteria, the low-voltage range for plant and mammalian cells. A double-pulse mode is intended to increase the life of electropores. All programming parameters can be input directly using the remote control keyboard.

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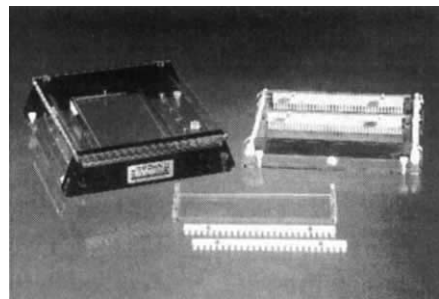
Reader Service No.8

Reader Service No.12

Stuart Scientific's SA 4 Vibro-Gene shaker, which combines the **action of a vortex mixer and shaker in one device**, can accommodate 0.5- and 1.5-ml conical or other small tubes with samples as small as 10 µl (*Reader Service No. 117*). The device's vibrating bar is made of rubber and has V-shaped slots cut into it; it vibrates at 2,500 oscillations per minute. Sample tubes are held with the lower portion of the tube located in a slot or against the vibrating bar. An illuminated window located under the vibrating bar allows the operator to view the samples against a bright background. Weighing just 3 kg, the device can be used on 220/240 V, 50 Hz.

The compact, Shimadzu UV1201 bench-top spectrophotometer, available from V.A. Howe, can be used for the **automatic calculation of DNA concentration** (*Reader Service No. 118*). To increase the flexibility of the instrument, an additional program pack is available for the quick-and-easy calculation of DNA and protein concentration. The software allows the standard 260 nm, 280 nm and 320 nm calculation values or further user-defined values to be entered.

Jordan Scientific's JSB-96 ultrawide Mini Gel-o-Submarine **minigel system** enables the user to run large numbers of samples, such as those generated by PCR analyses, plasmid screening and other techniques (*Reader Service No. 119*). The device can



Minigel system runs up to 100 samples.

run up to 100 samples in a single gel, allowing the user to analyse all 96 samples from a microtitre plate in as little as 90 min, the company says. Special combs are designed for quick loading of the sample with 8- or 12-channel pipettors. The minigel system is supplied with Jordan Scientific's QuickCast system, which is designed to provide a means of casting gels evenly without using tape. A selection of interchangeable combs and gel beds further extends the versatility of this system.

Appropriate Technical Resources has introduced the DNA Plus System and the DNA Mini System — compact and complete **vacuum concentration systems** dedicated for drying/concentrating RNA/DNA precipitates (*Reader Service No. 120*). The DNA Plus System is an all-in-one system comprising of a VR-minicentrifuge, a teflon-

coated membrane vacuum pump, a cooling trap with temperature to -60°C, and a built-in controller for automatic operation which includes a read-out of pressure, a timer, vacuum regulator and a start/stop function. The DNA Mini System is being billed as an environmentally-friendly, compact, space-saving design. It consists of a VR-minicentrifuge, a teflon-coated membrane pump, rotor, and built-in analog vacuum read-out and autovent.

■ Reagents/software

Following the recent acquisition of United States Biochemical by Amersham International, it is now possible to purchase all sequencing products from one supplier. A **DNA sequencing catalogue** is now available that, in addition to providing a complete product listing and background product information, offers application guides that are designed to help users overcome some of the common problems experienced in DNA sequencing (*Reader Service No. 121*).

Cambio are marketing a diagnostic method that has been developed to allow **direct analysis of single base mutation by polymerase chain reaction (PCR)** (*Reader Service No. 122*). Designed by PNA Diagnostics A/S, the method utilizes the discovery that peptide nucleic acids (PNAs) recognize and bind to their complementary nucleic acid sequences with high thermal stability and specificity. A PNA/DNA complex can effectively block the formation of a PCR product when the PNA is targeted either against the PCR primer site or an intervening sequence between the two PCR primers. This prevents the PCR primer from binding to its target, or it causes the polymerase to 'fall' off, both cases allowing selective amplification or suppres-



Sequencing aids.

sion of target sequences that differ by only one base pair.

A new application note is available from E. Merck that describes an interesting application for Benzonase, the company's un-specific **endonuclease**, in two-dimensional (2-D) gel electrophoresis (*Reader Service No. 123*). In it, the work of H. G. Meinrath (University of Hohenheim, Germany) is described, who reports improved two-dimensional gel electrophoresis and quantification of proteins after removal of nucleic acids with Benzonase.

With the newly expanded program in PC/GENE 6.8 from IntelliGenetics users can **generate circular maps of restriction enzyme digests** (*Reader Service No. 124*). Choose a set of restriction enzymes from the REBASE database of restriction enzymes, then automatically perform the restriction digest. Enzyme cut sites appear on a circular map, which includes an adjustment tool for repositioning the names of enzymes with a click of the mouse. This circular mapping capability is designed to assist in designing vectors and subclones, excising inserts for hybridization probes or mutagenesis studies, and preparing publication-quality graphics. PC/GENE 6.8 also includes new programs for identifying coding regions and coiled-coil regions. The new COD_JMC program can be used to locate potential exons in a mammalian nucleic acid sequence. COD_JMC predicts potential coding regions by comparing k-tuple frequencies in a sequence to those compiled from known exon and intron sequences. COIL is a new program that scans a protein sequence for the presence of potential coiled-coil regions. It reports the score for each residue in the sequence, and the probability that the residue is part of a coiled-coil formation. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For further details on any of the products, fill in the reader service card bound inside the journal. PCR is covered by patents owned by Hoffmann-La Roche.

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